In Vitro Cytotoxic Potential of Essential Oils of *Eucalyptus benthamii* and Its Related Terpenes on Tumor Cell Lines

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*Eucalyptus* L. is traditionally used for many medicinal purposes. In particular, some *Eucalyptus* species have currently shown cytotoxic properties. Local Brazilian communities have used leaves of *E. benthamii* as a herbal remedy for various diseases, including cancer. Considering the lack of available data for supporting this cytotoxic effect, the goal of this paper was to study the in vitro cytotoxic potential of the essential oils from young and adult leaves of *E. benthamii* and some related terpenes (α-pinene, terpinen-4-ol, and γ-terpinene) on Jurkat, J774A.1 and HeLa cell lines. Regarding the cytotoxic activity based on MTT assay, the essential oils showed improved results than α-pinene and γ-terpinene, particularly for Jurkat and HeLa cell lines. Terpinen-4-ol revealed a cytotoxic effect against Jurkat cells similar to that observed for volatile oils. The results of LDH activity indicated that cytotoxic activity of samples against Jurkat cells probably involved cell death by apoptosis. The decrease of cell DNA content was demonstrated due to inhibition of Jurkat cells proliferation by samples as a result of cytotoxicity. In general, the essential oils from young and adult leaves of *E. benthamii* presented cytotoxicity against the investigated tumor cell lines which confirms their antitumor potential.

1. Introduction

*Eucalyptus* L. is a large genus of the Myrtaceae family that includes some 900 species and subspecies [1]. These evergreen tall trees are native to Australia and show a worldwide distribution. For over 60,000 years ago, Australian aborigines developed a sophisticated empirical understanding of indigenous plants such as *Eucalyptus*. They traditionally used *Eucalyptus* leaves to heal wounds and fungal infections [2]. Although their pharmacological or toxicological properties have not been thoroughly investigated, infusions and decoctions of *Eucalyptus* plants are widely used in the treatment of respiratory diseases, for example, common cold, influenza, and sinus congestion [3, 4]. In Africa, the powder of barks has been indicated as insecticide. The leaves of *E. globulus* and *E. robusta* have been recommended for treating dysentery, articular pain, and tonsillitis in China. Besides their uses in folk medicine, many studies demonstrated analgesic, expectorant, anti-inflammatory, and antimicrobial properties from leaves of *Eucalyptus* spp. [3, 4].

Currently, extracts and components isolated from some *Eucalyptus* species have shown to possess cytotoxic activities. Cladocalol, a formylated triterpene, was isolated from
leaves of *E. cladocalyx* and showed cytotoxic effect on the myeloid leukemia cell line HL-60 [5]. A phlorogluconol
monoterpenic derivative, euglobal-G1, obtained from leaves of *E. grandis* exhibited a remarkable inhibitory effect on
two-stage carcinogenesis test of mouse skin tumors induced
by 7,12-dimethylbenz[a]anthracene [6]. Three new phenol
glycosides acylated with (+)-oleuropitic acid, cypellocarpins
A, B, and C, along with seven known compounds, isolated
from leaves of *E. cypellocarpa* suppressed an *in vivo*
two-stage carcinogenesis induced with nitric oxide and
12-O-tetradecanoyl-phorbol-13-acetate on mouse skin [7]. Ashour
[8] verified that essential oils from stems of *E. torquata* and
leaves of *E. sideroxylon* showed cytotoxic activities on MCF7
cells. Al-Fatimi et al. [9] investigated 14 plant species used as
traditional medicine in Yemen for cytotoxic activity against
human ECV-304 cells and reported that *E. camaldulensis* had
a remarkable biological activity. These studies increase the
interest in investigating the cytotoxic effect against tumor
cells from other species of *Eucalyptus* with the purpose of
improving the therapeutic opportunities against cancer.

*Eucalyptus benthamii* Maiden et Cambage is a tall attrac-
tive smooth white-barked tree, commonly known as camden
white gum or Nepean River gum. In Australia, it is listed as a
vulnerable species [10]. This species was recently introduced
in Southern Brazil as renewable source of timber due to
fast growing and high resistance to cold [11]. Regarding
the well-known medicinal properties of *Eucalyptus* species,
local communities from Campos Gerais region of Paraná in
Southern Brazil have used *E. benthamii* as an herbal remedy
for many therapeutic purposes, for example, microbial
infections and asthma. In spite of these uses, young and adult
leaves of *E. benthamii* have been currently indicated as a folk
practice for treating cancer [12]. In addition, this species has
been taken as tea obtained through infusing its leaves in hot
water or used as steam inhalations. Infusion is particularly
recommended for throat, esophageal, and stomach cancers
as well as lymphoma and cervical cancer. For lung cancer,
family farmers and their communities are deeply inhaling
the fumes resulting from the essential oil of *E. benthamii*
[12]. However, medicinal investigations about the essential
oil of *E. benthamii* are even lacking particularly related to
its possible cytotoxic properties. A recent paper reported
that the essential oil of *E. benthamii* provided larvicidal and
adulticidal activities against *Aedes aegypti* [13].

Considering this lack of available data for supporting the
cytotoxicity of the essential oil of *E. benthamii*, the goal of
this work was to investigate the *in vitro* cytotoxic activity of
the essential oil from young and adult leaves of *E. benthamii*
and some related terpene compounds, α-pinene, terpinen-
4-ol, and γ-terpinene on Jurkat (T leukemia cells), J774A.1
(murine macrophage tumor), and HeLa (cervical cancer)
cells lines.

### 2. Materials and Methods

#### 2.1. Plant Material.

Young and adult leaves of *E. benthamii*
were collected at Fazenda de Transferência de Tecnologia de
Embrapa Florestas (altitude: 926 m, latitude: 25° 10’ 09” S
and longitude: 50° 03’ 44” W) in Ponta Grossa, PR, Brazil,
during the summer of 2010. The species was identified by
the vouchers 59440 and 350231 and stored at the herbaria
from the Biological Sciences Center in the Federal University
of Paraná and Municipal Botanical Museum, respectively.

#### 2.2. Extraction of Essential Oil and GC-MS Analysis.

In separate, young and adult leaves of *E. benthamii* were air dried
and then distilled using a Clevenger type apparatus for 6 h. The essential oils were dried with anhydrous sodium sulphate
and stored in glass vial with Teflon-sealed caps at 4 ±
0.5°C in the absence of light until used. The identification
of volatile constituents was performed using a Hewlett-
Packard 6890 gas chromatography, equipped with a Hewlett-
Packard 5975 mass selective detector and capillary column
HP-5 (30 m × 0.25 mm × 0.25 μm). GC-MS was carried
out using split/splitless injection, with injector set at 220°C,
column set at 60°C, with heating ramp of 3°C/min and final
temperature at 240°C, and the detector was set at 250°C.
Helium was used as carrier gas at 1 mL/min. The GC-MS
electron ionization system was set at 70 eV. Quantitative
analysis was carried out using a Hewlett-Packard 5890 gas
chromatography equipped with a flame ionization detector
under the same conditions previously described. A sample
of each essential oil was dissolved in ethyl acetate (20 mg/mL)
for the analyses. Retention indices (RI) were determined by
injection of hydrocarbons standards and essential oil sample
in the same conditions. The oils components were identified
by comparison with data from literature [14] and the profiles
from the mass spectra libraries (Wiley 139, 275, and 7 and
Nist 127). The GC-FID quantification was obtained using
GC-FID chromatogram and was expressed as mean from
three samples of each extracted essential oil.

#### 2.3. Samples for Cell Culture Tests.

The previously obtained essential oils from young and adult leaves of *E. benthamii*
and its related terpenes: (+)-α-pinene, (−)-terpinen-4-ol and γ-terpinene were used for cell culture protocols. These
isolated compounds were purchased from Sigma as analytical
standard grade. A stock solution (100 mg/mL) of each sample
was prepared with propylene glycol and ethyl alcohol (1:4)
as solubilizing procedure [15]. Prior to the cell experiments,
these samples were diluted to final concentrations of 3, 10,
30, 100, and 300 μg/mL [16, 17] using culture medium.

#### 2.4. Cells and Cell Cultures.

Jurkat (T leukemia cells), J774A.1 (murine macrophage tumor), and HeLa (cervical
cancer) cells lines were obtained from American Type Cul-
ture Collection. All cultures were maintained in a color-free
medium composed of RPMI-1640 Medium (Sigma). This
medium was supplemented with 10% fetal bovine serum
(FBS, Life Technologies) and containing 0.1% of antibiotic
mix: 10,000 units penicillin and 10 mg streptomycin per mL
(Sigma). Sodium bicarbonate (2 mg/mL) was also added.
Cultures were maintained at 37°C in a humidified 5% CO₂
incubator. Experiments were performed at concentrations
of 250,000–500,000 cells per mL, and cells were in exponential
growth phase at the time of testing. These cells were
subcultured every 3-4 days. The viability of the cells exceeded 95% as determined by the trypan blue (0.4% trypan blue solution, Sigma) dye exclusion method.

2.5. In Vitro Cytotoxicity Tests

2.5.1. MTT Assay. The cytotoxicity was carried out by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay for investigating changes in mitochondrial/non-mitochondrial dehydrogenase activity [18]. In brief, cells (Jurkat, J774A.1 or HeLa cells lines, 5 × 10^3 cells/mL) were seeded on 96-well plates and cultured in RPMI 1640 containing 10% FBS at 37 °C. After incubation, the supernatant was removed, and MTT solution (0.5 mg/mL) was also added to each well 30 min prior to the end of the experiment. Water-insoluble dark blue formazan crystals formed in viable cells were solubilized in DMSO, and the absorbance was measured at 550 nm using a microplate reader (Biotek μQuant). Cell survival was determined by comparing the absorbance values obtained for treated and untreated cells. The cytotoxicity was expressed as the concentration of sample that inhibited 50% of cell growth (IC50) and was calculated by Probit regression.

2.5.2. Lactate Dehydrogenase (LDH) Activity Assay. In order to evaluate the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) released from the cytosol when cells were damaged or under stress, Jurkat cells (10^6 cells) were seeded on 24-well plates [20]. After 4 h, enzymatic measurements of LDH released into the supernatant were spectrophotometrically performed at 340 nm [19]. Absorbance values were then correlated with the number of viable cells to predict the cytotoxic activity. Serum-free culture medium and 1% TritonX-100 (Sigma) were used as negative and positive controls, respectively.

2.5.3. Analysis of Cell DNA Content. The effect of samples on cell proliferation activity was determined by measuring DNA content. Jurkat cells were seeded (1.5 × 10^5 cells/mL) on 24-well plates [20]. After 24 h, each sample at 300 μg/mL concentration was added. After 4 h, the absorbance was measured at 570 nm using a microplate reader (Biotek μQuant). Cell survival was determined by comparing the absorbance values obtained for treated and untreated cells. The cytotoxicity was expressed as the concentration of sample that inhibited 50% of cell growth (IC50) and was calculated by Probit regression.

2.6. Statistical Analyses. Results were evaluated by Prism software. Statistical analyses were carried out by one way ANOVA (Graph Pad Prism 5.01 Software) followed by Tukey post hoc test.

3. Results and Discussion

The chemical composition of the essential oils from young and adult leaves of E. benthamii is presented in Table 1. Both volatile oils consisted of a complex mixture of monoterpenes and sesquiterpenes. The main identified compounds for the essential oil from young leaves (YLEO) of E. benthamii were α-pinene (36.82%), globulol (20.54%), aromadendrene (15.94%), and γ-terpinene (5.51%). The oil extracted from adult leaves (ALEO) was composed mainly by α-pinene (36.92%), globulol (20.22%), aromadendrene (12.40%), and γ-terpinene (4.38%). Therefore, the volatile compositions were quite similar regarding these main compounds. However, some differences on quantitative composition of these essential oils were particularly related to their minor compounds which can be explained by genotype conditions. It has been extensively reported that many Eucalyptus species show heteroblasty, producing juvenile and adult leaves differing markedly in morphology and anatomy [21]. Consequently, differences on the biosynthetic pathway can also occur and lead to a broad range of representative reaction types of terpenoid metabolism [22] which influence the final volatile composition.

Some previous papers were devoted to study the volatile chemical composition of essential oils provided by E. benthamii. Tian et al. [23] verified that α-pinene (31.00%), globulol (15.34%), aromadendrene (13.80%), and epiglobulol (4.86%) were the principal constituents of the essential oil extracted from leaves of E. benthamii by steam distillation. Silva et al. [24] investigated the percentage of α-pinene provided by the essential oil of E. benthamii during the seasons and observed values varying from 24.2% (spring) to 47.6% (fall). Mossi et al. [25] aimed at evaluating the insecticidal and repellency effect of five essential oils of Eucalyptus against Sitophilus zeamais Motschulsky (Coleoptera, Curculionidae) and reported that the volatile oil of E. benthamii contained α-pinene (54.04%), viridiflorol (17.12%), 1,8-cineole (9.93%), aromadendrene (7.3%), and globulol (3.61%). Lucia et al. [13] studied the fumigant and larvicidal activity of some essential oils of Eucalyptus against Aedes aegypti (Diptera, Culicidae) and showed that the essential oil of E. benthamii var. benthamii exhibited a higher content of α-pinene (73.15%) while the essential oil of E. benthamii var. dorrigoensis revealed 1,8-cineole (74.73%) as the major component. Therefore, the volatile chemical compositions reported in the present paper for the studied essential oils from young and adult leaves of E. benthamii are in accordance to the literature due to their relatively high concentrations of α-pinene. The differences in chemical composition can be related to soil and climate conditions, water stress, collection place, nutrition, and other abiotic factors. Moreover, the presence of subspecies and chemotypes can lead to changes in the final volatile chemical composition of the essential oil of E. benthamii. Thus the evidence of these qualitative and quantitate differences reinforces the need for
establishing the chemical profile of this essential oil prior to its biological assay.

Furthermore, *E. benthamii* also revealed some particular differences in the chemical composition as compared to usual *Eucalyptus* species, since of its essential oils contained only traces of 1,8-cineole. However, many other species of *Eucalyptus* which do not contain 1,8-cineole as the major volatile compound have been revealed some remarkable pharmacological activities. In that sense, Elaissi et al. [26] screened the antibacterial activities of twenty essential oils of *Eucalyptus* species. The volatile oil of *E. odorata*, that showed less than 5% of 1,8-cineole, demonstrated the best inhibition zone diameter against *S. aureus*. Although 1,8-cineole is usually related to the treatment of respiratory diseases, other volatile components provided by some essential oils from *Eucalyptus* as camphene, globulol, limonene, *α*-pinene, *β*-pinene, and *p*-cymene have been provided some properties as antitussives and expectorants [27]. Therefore the fact that many of the therapeutic effects of the essential oils from *Eucalyptus* spp. that have been attributed to 1,8-cineole do not determine that other species that contain 1,8-cineole in trace amounts have not been used as herbal medicine.

The results for MTT assay are summarized in Tables 2 and 3 considering exposure periods of 24 and 72 h, respectively. In general, the volatile oils from young and adult leaves of *E. benthamii* showed some degree of cytotoxicity against the studied cells. Regarding the essential oil provided by young leaves (YLEO) of *E. benthamii*, Jurkat cells revealed a more sensitive response (IC$_{50} = 108.33$ μg/mL at 24 h and IC$_{50} = 56.51$ μg/mL at 72 h) when compared to J77A.1 cells (IC$_{50} = 287.98$ μg/mL at 24 h and IC$_{50} = 166.87$ μg/mL at 72 h). The essential oil obtained from adult leaves (ALEO) of *E. benthamii* demonstrated the same behavior for these two cell lines (Tables 2 and 3). Similar data were also observed for *α*-pinene, *γ*-terpinene, and terpinen-4-ol in which Jurkat cells had a more sensitive response to these compounds (IC$_{50} = 192.42$, 136.60, and 50.20 μg/mL at 24 h and IC$_{50} = 186.09$, 156.92, and 54.84 μg/mL at 72 h, resp.) than J77A.1. The terpenes *α*-pinene and *γ*-terpinene showed no activity against J77A.1, while terpinen-4-ol revealed 220.02 and 189.70 μg/mL as IC$_{50}$ value at 24 and 72 h, respectively. Regarding the cytotoxicity against HeLa cells, the volatile oils from young and adult leaves of *E. benthamii* showed IC$_{50}$ of 84.24 and 110.02 μg/mL at 24 h and 120.57 and 101.90 μg/mL at 72 h, respectively. It was verified that *α*-pinene, *γ*-terpinene and terpinen-4-ol did not exhibit effect on this tumor cells. As proposed by previous studies [16] that performed the cytotoxic effect of essential oils, IC$_{50}$ values between 10–50 μg/mL represent a strong cytotoxic activity. Moreover, IC$_{50}$ values between 50–100, 100-200, and 200-300 μg/mL indicate moderate, weak, and very weak cytotoxic properties, respectively. Furthermore IC$_{50}$ values higher than 300 μg/mL represent no cytotoxicity.

Considering the cytotoxic activity on the three studied tumor cells based on MTT assay, the essential oils demonstrated enhanced results than *α*-pinene and *γ*-terpinene, which do not contain 1,8-cineole as the major volatile components.

### Table 1: Chemical composition of the essential oil from young and adult leaves of *E. benthamii*.

| Volatile compound       | RT   | RI   | YLEO (%) | ALEO (%) | Identification   |
|-------------------------|------|------|----------|----------|-----------------|
| *α*-pinene              | 4.90 | 935  | 36.82    | 36.92    | RI, MS          |
| *β*-pinene              | 5.90 | 977  | 1.59     | 1.90     | RI, MS          |
| *p*-cimene             | 7.27 | 1024 | 0.81     | 0.90     | RI, MS          |
| Limonene                | 7.39 | 1028 | 5.51     | 4.38     | RI, MS          |
| *γ*-terpinene           | 8.38 | 1058 | 11.29    | nd       | RI, MS          |
| (*E*)-pinocarveol       | 11.29| 1138 | nd       | 0.84     | RI, MS          |
| terpinen-4-ol           | 12.82| 1177 | 1.23     | 1.05     | RI, MS          |
| *α*-terpinol           | 13.39| 1192 | 1.32     | 2.02     | RI, MS          |
| *α*-gurjunene          | 22.25| 1406 | 1.70     | 1.29     | RI, MS          |
| Aromadendrene          | 23.50| 1437 | 15.94    | 12.40    | RI, MS          |
| *α*-aromadendrene      | 24.30| 1457 | 1.98     | 1.48     | RI, MS          |
| Viridiflorene           | 25.69| 1492 | 1.47     | 1.13     | RI, MS          |
| Globulol                | 29.25| 1584 | 20.54    | 20.22    | RI, MS          |
| Viridiflorol            | 29.47| 1590 | 1.63     | 3.13     | RI, MS          |
| Rosifolius              | 29.84| 1599 | 1.63     | 1.60     | RI, MS          |
| 10-*γ*-eudesmosl        | 30.59| 1620 | 1.78     | 1.64     | RI, MS          |

| Compounds identified       | YLEO (%) | ALEO (%) |
|-----------------------------|----------|----------|
| Monoterpene hydrocarbons    | 44.73    | 44.58    |
| Oxygenated monoterpene      | 2.55     | 3.91     |
| Sesquiterpene hydrocarbons  | 21.09    | 16.30    |
| Oxygenated sesquiterpenes   | 25.58    | 26.59    |

Legend: YLEO: young leaves essential oil; ALEO: adult leaves essential oil; RT: retention time; RI: retention index; MS: mass spectroscopy; nd: not detected.
from stems of adenocarcinoma cell line (MCF7). The essential oil extracted of volatile oils and extracts from stems, leaves, and flowers of Eucalyptus torquata exhibited cytotoxicity against some human cancer cell lines (A549, MCF-7, Caco-2, HL-60, and K562) and a mouse cell line (B16F10). Regarding some human cancer cell lines, E. benthamii from young and adult leaves of Sideroxylon cinalis L. showed a cytotoxic activity against cancer cell lines (A549, MCF-7, Caco-2, HL-60, and K562) and a mouse cell line (B16F10). Regarding the cytotoxic effect of essential oils of Eucalyptus, data are remarkable restricted. Ashour [8] showed cytotoxic activities of volatile oils and extracts from stems, leaves, and flowers of E. sideroxylon and E. torquata against the human breast adenocarcinoma cell line (MCF7). The essential oil extracted from stems of E. torquata exhibited cytotoxicity against MCF7 cells followed by volatile oils from leaves of E. torquata and leaves of E. sideroxylon.

Although the studied essential oils showed cytotoxic results against tumor cell lines, its major component α-pinene did not demonstrate the same behavior with only a weak response as an isolated cytotoxic agent against Jurkat cells. This result is a further evidence that the combination of volatile components of essential oils can influence the final cytotoxic effect. No cytotoxicity was observed when α-pinene was evaluated against J774A.1 and HeLa. This monoterpene is widely related to antibacterial and insecticide activities and can be used for industrial purposes in camphor synthesis and perfumery products [33, 34]. The monoterpene α-pinene has been exhibited in vitro cytotoxicity on HEPG2 human hepato-cellular carcinoma cells [35]. The in vitro cytotoxicity of the essential oil and major constituents of Cymbopogon jwarancusa (Jones) Schult. demonstrated a percentage of inhibition less than 20% of THP-1 (human acute monocytic leukemia), A-549 (adenocarcinomic alveolar basal epithelial), HEP-2 (human liver tumor), and IGR-OV-1 (ovarian carcinoma) cell lines by α-pinene (100 μg/mL) [36]. Another study reported that α-pinene isolated from Schinus terebinthifolius Raddi induced apoptosis and conferred antimetastatic protection in a melanoma model. It has been shown that α-pinene, while inactive alone against C32 (human amelanotic melanoma) and ACHN (human renal cell adenocarcinoma) cells, can act in synergy with other antiproliferative components of essential oils [37]. Zhou et al. [38] clarified that α-pinene inhibits the nuclear translocation of NF-κB which regulates the expression of genes that play critical roles in apoptosis and immunomodulation. In spite of many papers about α-pinene and its cytotoxicity against tumor cells, none of them was related to Jurkat, HeLa, and J774A.1 cell lines.

The monoterpene hydrocarbon y-terpinene presented some cytotoxic properties against Jurkat cell line. However, it was not observed any cytotoxicity for this volatile compound.

### Table 2: Evaluation of cytotoxicity by MTT assay in cell lines after 24 h.

| Cell line | YLEO | ALEO | IC50 (μg/mL) α-pinene | y-terpinene | terpinen-4-ol |
|-----------|------|------|------------------------|-------------|---------------|
| Jurkat    | 108.33 ± 1.83 | 54.96 ± 5.80 | 192.42 ± 9.38 | 136.6 ± 10.67 | 50.20 ± 13.03 |
| J774A.1   | 287.98 ± 3.54 | 252.55 ± 1.91 | >300 | >300 | 220.02 ± 7.15 |
| HeLa      | 84.24 ± 5.94 | 110.02 ± 2.89 | >300 | >300 | >300 |

Legend: YLEO: young leaves essential oil; ALEO: adult leaves essential oil; IC50: concentration that reduces the mitochondrial activity by 50%. The results are shown as mean ± SD from three independent experiments.

### Table 3: Evaluation of cytotoxicity by MTT assay in cell lines after 72 h.

| Cell line | YLEO | ALEO | IC50 (μg/mL) α-pinene | y-terpinene | terpinen-4-ol |
|-----------|------|------|------------------------|-------------|---------------|
| Jurkat    | 56.51 ± 1.48 | 36.63 ± 3.52 | 186.09 ± 37.98 | 156.92 ± 10.22 | 54.84 ± 3.85 |
| J774A.1   | 166.87 ± 5.77 | 178.85 ± 6.11 | >300 | >300 | 189.7 ± 14.74 |
| HeLa      | 120.57 ± 6.22 | 101.90 ± 19.47 | >300 | >300 | >300 |

Legend: YLEO: young leaves essential oil; ALEO: adult leaves essential oil; IC50: concentration that reduces the mitochondrial activity by 50%. The results are shown as mean ± SD from three independent experiments.
against J774A.1 and HeLa cell lines below 300 μg/mL. There are few studies linking γ-terpinene and cytotoxicity activity. It was verified cytotoxic effects for leukemia HL-60 and NB4 cells using the essential oil obtained from dried leaves of *Majorana hortensis* which showed a content of 15.0% γ-terpinene [39]. Bourgou et al. [40] studied the cytotoxic activity of γ-terpinene against human lung carcinoma A-549 and colon adenocarcinoma DLD-1 cells and achieved IC_{50} ≥ 100 μM (13.62 μg/mL) for both cell lines.

The isolated terpinen-4-ol showed a cytotoxic effect against Jurkat cells similar to the evaluated essential oils. This monoterpenes has been extensively related to antiviral, antibacterial, antifungal, and insecticidal effects as well as it has been shown antioxidant and anti-inflammatory activities [41–45]. This compound also exhibited antiproliferative and cytotoxic effects on murine AE17 mesothelioma and B16 melanoma tumor cell lines [46]. The essential oil of *Melaleuca alternifolia* and its main component, terpinen-4-ol, were able to impair the growth of human M14 melanoma cells [47]. Wu et al. [48] verified that terpinen-4-ol elicited a dose-dependent cytotoxic effect on human non-small cell lung cancer. Cytotoxicity of Australian tea tree oil (M. *alternifolia*), terpinen-4-ol, 1,8-cineole, and α-terpineol were investigated on five different human cell lines: HEPG2, HeLa, MOLT-4 (human acute lymphoblastic leukemia), K-562 (Human erythromyeloblastoid leukemia), and CTVR-1 (B cell-derived from bone marrow of a patient with acute myeloid leukaemia). The overall rating for cytotoxicity of tea tree oil and its components was α-terpineol > tea tree oil > terpinen-4-ol > 1,8-cineole [49]. Another study indicated that tea tree oil and its major component, terpinen-4-ol, can also interfere with the migration and invasion processes of drug-sensitive and drug-resistant melanoma cells [50]. Despite several papers about the cytotoxic potential of terpinen-4-ol on cancer cells and the obtained results against Jurkat cells, this investigation reported weak/very weak cytotoxicity of terpinen-4-ol against J774A.1 and no cytotoxicity against HeLa cells lines.

Considering all previous results for MTT assay, Jurkat cells were chosen for further evaluation using LDH activity assay and analysis of cell DNA content in order to elucidate the possible mechanism of cytotoxicity.

The Figure 1 shows the cytoplasmic LDH released of Jurkat cells treated with each sample at 300 μg/mL concentration. Legend: YLEO: young leaves essential oil; ALEO: adult leaves essential oil; positive control: 1% TritonX-100. The results are shown as mean ± SD from three independent experiments. The symbol *** represents a value of *P* < 0.001 that was considered to be highly significant compared to the positive control.

The Figure 2: Results of DNA content of Jurkat cells treated with each sample at 300 μg/mL concentration. Legend: YLEO: young leaves essential oil; ALEO: adult leaves essential oil; positive control: vincristine (40 nM). The results are shown as mean ± SD from three independent experiments. The symbols *** and ### represent a value of *P* < 0.001 that was considered to be highly significant compared to the negative and positive controls, respectively.
Considering the few number of investigations about cytotoxic effects of *Eucalyptus* spp. on tumor cells, this paper reported that essential oils from young and adult leaves of *E. benthamii* present cytotoxicity mainly against Jurkat and HeLa cell lines in comparing to the isolated terpenes, particularly α-pinene and γ-terpinene. The obtained results also demonstrate the importance of the *E. benthamii* as an alternative herbal source of a complex mixture of volatile compounds that can be used as cytotoxic agent.

Although the essential oils provided by *Eucalyptus* species have been used in folk medicine, it is important to mention that their use must be cautious because a systemic toxicity can occur from ingestion or topical application at higher doses as widely reported [51–54]. The probable lethal dose of pure essential oil of *Eucalyptus* spp. for an adult is in the range of 0.05 mL to 0.5 mL/kg, and severe poisoning has occurred in children after ingestion of 4 to 5 mL.

### 4. Conclusion

In general, these findings demonstrated that the essential oils of *E. benthamii* show improved cytotoxic potential than the isolated terpenes α-pinene and γ-terpinene. Moreover, the obtained results support an experimental basis for reporting that the essential oils of *E. benthamii* lead to cell death mostly by apoptotic process. Furthermore, these data can also pave the way for future development of therapeutic opportunities against cancer.

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