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Eucalyptus cinerea: Microscopic Profile, Chemical Composition of Essential Oil and its Antioxidant, Microbiological and Cytotoxic Activities

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HIGHLIGHTS

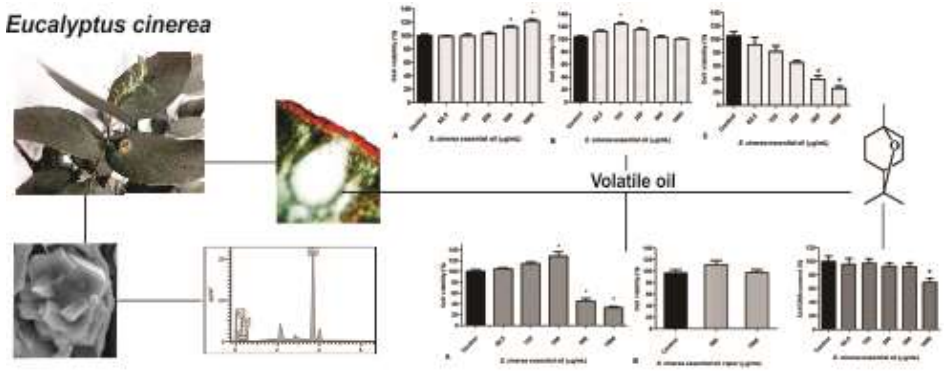
- *Eucalyptus cinerea* essential oil (EO) contains 1,8-cineole as the main component.
- This EO demonstrated antioxidant and antimicrobial properties.
- This EO reduced the viability of Jurkat and Calu-3 cells and decreased DNA content.
- This EO had cell death mechanism related to apoptosis.

Abstract: *Eucalyptus* species possess anti-inflammatory, antifungal, antibacterial, and insecticidal properties. In this study, the chemical composition and biological activities of *Eucalyptus cinerea* essential oil

(EO) and the leaf and stem anatomy were investigated. EO was extracted by Clevenger apparatus and the compounds were identified by GC/MS. The antioxidant activity was evaluated by DPPH, ABTS, and reducing phosphomolybdenum complex. Broth microdilution was used to determine antimicrobial activity. Cytotoxicity was verified against HeLa, HRT-18, and Calu-3 cells by MTT assay. The cytotoxic mechanism was studied by cell DNA content, cell cycle, and DNA fragmentation. The microscopic analyzes of the leaves and the stems were performed by light microscopy, field emission scanning electron microscopy, and energy-dispersive X-ray spectroscopy. The main constituent of the EO was 1,8-cineole (55.24%). The EO showed low antioxidant and antimicrobial activities. Calu-3 cells showed a significant reduction in viability with IC_{50} of 689.79 ± 29.34 µg/mL. EO at 1000 µg/mL decreased the DNA content in Jurkat cells. In general, EO increased cell percentage in sub-G0 and S phases with concomitant reduction of cell percentage in G0/G1 and G2/M phases and provided DNA fragmentation of 29.73%. Anatomical and micromorphological features of the leaves and stems can help in the species identification and its differentiation from other *Eucalyptus* species.

Keywords: anatomy; biological activity; histochemistry; microscopy; Myrtaceae; terpenoids; volatile oils.

GRAPHICAL ABSTRACT



INTRODUCTION

Eucalyptus L'Hér. is one of the largest genera of Myrtaceae, represented with about 800 species. Most of these species are native to Australia and are widely cultivated throughout the world [1]. Various *Eucalyptus* species have particular medicinal properties and are also used in the production of paper, timber, honey, and essential oil [1,2].

Eucalytpus species have been shown to possess several medicinal properties, such as antiseptic, antioxidant, antimicrobial, acaricidal, insecticidal, and herbicidal activities, and presented a significant number of chemical compounds in the essential oils. These characteristics can be useful in the rationalization of the use of species of *Eucalyptus*, especially because the volatile compounds are used in agrochemical and pharmaceutical applications as well as food preservatives and flavorants [1,3]. Other therapeutic activities, such as anti-inflammatory, astringent and healing properties, have also been reported for *Eucalyptus* species [3–6].

Eucalyptus has become a major non-native crop in Brazil, with plantations taking up 5.63 million hectares [7], making the country the largest grower of *Eucalyptus* followed by India (4.3 m ha) and China (2.6 m ha). In Brazil, *Eucalyptus cinerea* F.Muell. ex Benth. is commonly called *eucalipto-cinzento*, *eucalipto-azul* and *eucalipto-argentino*. It is a subtropical species found in the latitudes 33ºS to 36ºS of the coastal region and central part of the Australian states of New Wales of the South and Victoria, between 100 to 1300 m of altitude and is well acclimatized in Brazil. It is a large tree with a rough, persistent, fibrous, and grayish bark. The leaves are sessile and measure 3.5-6.5 cm long and 3-4.5 cm wide. They are oval, with acuminate apex, rounded base, entire margin, and intense reticulate veins [1].

The essential oils (EOs) of *E. cinerea* have presented anti-inflammatory [8] and antimicrobial activities against *Staphylococcus aureus*, *S. epidermidis, Escherichia coli*, *Pseudomonas aeruginosa, Candida albicans* [9]*, Listeria ivanovii* and *Bacillus cereus* [10]. EO may have potential applications in food and pharmaceutical products [10]. Considering the good yield and the high 1,8-cineole content in the EO, *E. cinerea* is a less exploited species [9] and could be utilized as an alternative source for 1,8-cineole production [11]. 1,8-Cineole is commonly used in the medicinal and perfumery industries [11].

Hence, the present work was aimed to (1) dereplicate the chemical composition of the EOs; (2) to verify the antioxidant and antimicrobial activities; (3) to characterize the cytotoxic potential of the EOs against HeLa, HRT-18, and Calu-3 cells lines in addition to confirm the cytotoxic mechanism by morphology feature changes, and (4) to investigate the anatomical and histochemical characteristics of the leaf and stem to provide botanical data to support the identification of *E. cinerea*.

MATERIAL AND METHODS

Plant material

Fresh samples of the leaves and stems of *Eucalyptus cinerea* were collected during November 2015 from the plants growing in Ponta Grossa (latitude 24°18′ S and longitude 49°37′ W), Paraná, Brazil. At least three samples of mature leaves obtained from the fourth node and below, and stem fragments collected 5-15 cm from the shoot tips, were prepared for extraction of essential oil and microscopy analyzes. A flowering branch of *Eucalyptus cinerea* was used to prepare a voucher specimen, which was stored in the Herbarium of the State University of Ponta Grossa under the number 21260 HUPG. This material was identified using relevant literature [1,12–14]. Access to the botanical material was approved by Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) under code AE53EDF.

Extraction of essential oil (EO) and GC-MS analysis

For the extraction of EO, the leaves and stems of *E. cinerea* were prepared as described by Budel and coauthors [15]. For the chemical profiling of the EO, the methods defined by Saulle and coauthors [16] were used.

Antioxidant activity

Free radical scavenging activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH•) test

The scavenging activity of *E. cinerea* EO for DPPH• was measured as described by Yen and Wu [17], and Chen and coworkers [18]. Briefly, EOs and the positive controls (rutin and gallic acid) in methanol at 10; 15; 20; 25 mg/mL were investigated. The methanol was employed to obtain a DPPH solution (0.1mM). It was transferred to 20 µL of the EO solution of *E. cinerea* as well as the positive control and blank (methanol) and 100 µL of the DPPH solution in 96 well microplates. The reading was performed in a microplate reader (Biotek Instruments, Winooski, VT, USA) at 517 nm directly after mixing and at the end of 30 min incubation in the dark at room temperature. The antioxidant activity was determined as a percentage and calculated by the decay rate of the DPPH solution absorbance with EO after 30 min of reaction. Thus, the antioxidant activity of the EO was expressed as the half-maximal inhibitory concentration (IC_{50}).

ABTS radical-scavenging activity

To acquire the radical cation $ABTS^{\bullet+}$, methods described by Re and coworkers [19] were performed. Then, the solution $ABTS\bullet^{+}$ was diluted in ethanol to obtain an absorbance value at ± 0.7 to 734 nm. The complexation between $ABTS\bullet^{+}$ and different EO concentrations (10; 15; 20; 25 mg/mL) was measured by decreasing absorbance at 734 nm after 30 min microplate reader (Molecular Devices, SpectraMax 190, Sunnyvale, USA). A rutin and gallic acid solution (10; 15; 20; 25 mg/mL) was used as standard. The results were expressed as the concentration of antioxidant needed to suppress 50% of the oxidizing agent (IC_{50}).

Reducing phosphomolybdenum complex

To define the antioxidant capacity, the phosphomolybdenum complex reduction method was used as described by Prieto and coauthors [20]. An aliquot of 300 µL of EO in an ethanol solution at 200 µg/mL was added to a test tube, along with 3 mL of the reagent (4 mmol/mL ammonium molybdate, 28 mmol/mL sodium phosphate, 0.6 mol/L sulfuric acid). Then, the tubes were hermetically sealed and transferred to a water bath at 95ºC during 90 min. The tube was sealed and transferred to a water bath at 95°C for 90 min. After cooling, the reading was performed at 695 nm against a blank (300 µL of ethanol was added 3 mL of reagent), using a spectrophotometer model UV/Vis Shimadzu-1601-695 nm. Ascorbic acid (Merck®) was considered as 100% antioxidant activity.

Antimicrobial activity

Broth microdilution method

The analyzes used strains from the American Type of Culture Collection (ATCC), *S. aureus* ATCC® 25923, *S. pyogenes* ATCC® 12228, *E. coli* ATCC® 25922, and *P. aeruginosa* ATCC® 27853. By the microdilution broth method proposed by NCCLS [21], bacteria were inoculated in BHI broth (brain heart infusion). A similar methodology was used for *Candida albicans* ATCC® 10231, substituting the culture medium with Sabouraud. Serial dilutions (0.013; 0.026; 0.052; 0.104; 0.208; 0.416 mg/mL) of *E. cinerea* EOs were added in each well and then 10 µL of each microbial inoculum were added. The microplates were covered and incubated at 35°C for 24 h.

Perio GARD® (0.12% chlorhexidine) was used as a positive control. To evaluate the viability of microorganisms in non-inhibitory concentrations, 50 µL of TCC dye (2, 3, 5 triphenyl tetrazolium chloride) at 1% was used and incubated at 35ºC for 30 min. After sowing, the Petri dishes were incubated at 37°C for 24 h. The results of the antimicrobial testing were expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Cytotoxic activity

Samples for cell culture tests

According to Virador and coauthors [22], a stock solution (300 mg/mL) with propylene glycol and ethyl alcohol (1:4) was prepared as a solubilizing process. Prior to the cell experiments, this sample was diluted to final concentrations of 62.5, 125, 250, 500, 1000 µg/mL for *E. cinerea* EO and 500, 1000 µg/mL for the vapor EO assay [23,24] using culture medium.

Cell and cell cultures

HeLa (uterine cervix adenocarcinoma), HRT-18 (colon adenocarcinoma), Jurkat (T leukemia cells), and Calu-3 (lung adenocarcinoma) cells were acquired from American Type Culture Collection (Rockville, MD, USA); ATCC® CCL 244™; Cell Bank of Rio de Janeiro (Rio de Janeiro, Brazil); and ATCC® HTB-55™, respectively. All the cultures were maintained in RPMI-1640 medium, which was added with 10% fetal bovine serum (FBS), comprising 10,000 units of penicillin and 10 mg of streptomycin/mL. The cultures were preserved at 37°C in a humidified 5% CO_2 incubator. These cells were subcultured every 3-4 days. The viability of the cells surpassed 95% as determined by the trypan blue (0.5% trypan blue solution) dye exclusion method.

In vitro cytotoxicity

Cytotoxicity of essential oil

Calu-3 cells (2.5×10^4 cells/mL), Jurkat (1×10^5 cells/mL), HeLa and HRT-18 (8×10^4 cells/mL) after 24 h plating was treated with *E. cinerea* EO in the following concentrations 62.5, 125, 250, 500 and 1000 µg/mL during 72 h and cytotoxicity was determined by the [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] (MTT) test.

Cytotoxicity of vapor of essential oil

To test the cytotoxicity of EO vapor, the Calu-3 cells were seeded at 5×10^4 cells/mL in 24 well plates, keeping wells devoid of cells where EO was solubilized at concentrations 500, 1000 µg/mL. The volatile components tested were volatilized at 37°C, at the standard temperature of the CO_2 greenhouse. The treatment was performed for 72 h, and the cytotoxicity was determined by the MTT assay. The control used contained the solubilization vehicle of EO (25% propylene glycol and 75% ethanol) that did not exceed 0.3% in culture [25].

MTT assay

MTT test was performed for investigating changes in mitochondrial/non-mitochondrial dehydrogenase activity as described by Seal and coworkers [25]. The cell lines were seeded on 24 well plates and cultured in RPMI 1640 containing 10% FBS at 37°C and 5% CO_2 for 24 h. Then, different concentrations of EO were

added. After incubation and proceeding to the end of the experiment, the supernatant was removed, and 0.5 mg/mL of MTT solution was added to each well. Water-insoluble dark blue formazan crystals formed in viable cells were solubilized in DMSO. The spectrophotometric absorbance was read at 550 nm in Biotek, µQuant® plate reader. To determine survived cells, the absorbance values acquired for the treated and untreated cells were considered. The cytotoxicity of EO was expressed as the concentration of the sample, which inhibited 50% of cell growth (IC_{50}) and was calculated by Probit regression using the Least Squares method.

Analysis of mechanisms involved in the cytotoxic activity

Analysis of cell DNA content: Propidium iodide (PI)

Propidium iodide (PI) assay was used to analyze the DNA content as described by Dengler and coauthors [26] with some modifications. Jurkat cells were seeded (1×10^5 cells/mL) on 24-well plates. After 24 h, different concentrations of *E. cinerea* EO (62.5; 125; 250; 500; 1000 µg/mL) were added. After 72 h, aliquots of 50 µL, corresponding to a cell concentration of 4×10^4 cells/mL, were transported to a black 96-well plate. Then, 150 µL of a solution containing 100 µg/mL PI, 0.2% (v/v) Triton X-100 and 0.2% (v/v) sodium citrate was added. After an incubation period of 24 h at room temperature, the fluorescence (FU) was measured using a Cytofluor H 4000 microplate reader (excitation at 525 nm and emission at 617 nm) to quantify the amount of attached viable cells.

Cell cycle and DNA fragmentation assay

According to Crissman and Steinkamp [27], Jurkat cells (1×10^5 cells/mL) were seeded on 24 well plates and cultured in RPMI 1640 containing 10% FBS at 37 °C and 5% CO_2 for 24 h. The cells were treated with different concentrations of *E. cinerea* EO (125, 250 and 500 µg/mL) for 72 h. The cells were centrifuged at 300 g for 7 min. The pellets were washed with PBS buffer (1 mL), centrifuged, resuspended in 400 µL of PSSI buffer (1.4 µL of 1%Triton X - 100; 20 µL of 20 mg/mL RNAse; 60 µL of 2 mg/mL propidium iodide and 8.520 µL of PBS). Each sample was incubated for 15 min at 37°C. The analysis was performed on the flow cytometer (FACsCANTO II from Becton & Dickinson, San Jose, CA, USA). The results corresponding to 30,000 events per sample were obtained and processed in the DIVA program (Becton & Dickinson) and expressed as percentage of cells in different phases of the cell cycle and fragmented DNA.

Microscopic procedure

The methods used for plant material preparation, light microscopy (LM), scanning electron microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDS), and histochemical tests were fully detailed in a previous paper by Machado and coauthors [28].

Statistical analysis

Statistical analysis was performed by ANOVA and Tukey's posthoc test. The results were expressed as mean ± standard error of the mean (SEM). A value of $p < 0.05$ was considered indicative of significance. All the tests were carried out using Graph Pad Prism 5.01 software.

RESULTS AND DISCUSSION

Yield and Chemical Composition of Essential Oil (EO)

The EO yield of *E. cinerea* was 5.4% (v/w). This species produces the highest quantity of EOs in the genus. Variations in the EO yield have been reported, such as 2.48% EO from the plants cultivated in Argentina [29], 4.5% for leaves and 0.5% for stems from the plants cultivated in Egypt [30] and 6.07% for those sourced from Paraná state in Brazil [9]. *Eucalyptus cinerea* EO frequently contains more than 80% of 1,8-cineole [2] as the main component. Comparing the studies from Argentina, Brazil and Tunisia, the presence of 1,8-cineole was between 56.9 – 88.5% [2]. However, a low concentration of 1.8-cineole (5.2%) was found in the EO of leaves from Congo.

In the present work, the monoterpene 1,8-cineole (55.24%) was identified as the major compound in the EO of *E. cinerea* (Table 1). Also known as eucalyptol, this monoterpene has medicinal properties and can be used as an anesthetic and antiseptic [31]. 1,8-Cineole is also used for the treatment of chronic bronchitis, sinusitis and respiratory infections, and for stimulating expectoration. This compound also has anti-

inflammatory action in patients with asthmatic bronchitis and presents more potential antimicrobial activity than citronellal and caryophyllene [32,33]. 1,8-Cineole has been identified as the major compound (22-83%) in several species of *Eucalyptus* [2].

Several factors can influence *Eucalyptus* EO production, including genetic variability, leaf age, environmental conditions, plantation management, part of the plant used for EO extraction, the extraction process, and analysis method [2,16,34]. According to Bugarin and coauthors [35], there is great variation in the composition of volatile compounds for the same species of *Eucalyptus* obtained from different geographic origins. The chemistry of the EOs must be better studied because biological activities are highly dependent on the EO chemical composition [2].

Silvestre and coworkers [36] have observed that young leaves tend to have a lower 1,8-cineole content. However, in Argentina, 90.7% of 1,8-cineole was found in fresh and young leaves of *E. globulus* subsp. *bicostata* (Maiden, Blakely & Simmonds) J. B. Kirkp., obtained in the first hour of distillation [37]. In that sense, the amount of 1,8-cineole is related to leaf age and the time of hydrodistillation. In the present study, adult leaves and stems were used for the extraction, and the EO was collected after 3 h of hydrodistillation.

α-terpinyl acetate was the second major compound in the EO of *E. cinerea* (21.64%). It is mainly used for medicinal and pharmaceutical purposes [38]. The monoterpenoids 1,8-cineole and α-terpinyl acetate in *Eucalyptus* species have important antimicrobial activities and are used as biomarkers for the plants' resistance against eucalyptus rust disease [39].

Table 1. Chemical composition of *Eucalyptus cinerea* essential oil.

Volatile compounds	RI cal.[2]	RI lit.[1]	Peak area (%)	Identification
α-pinene	932	932	7.94	RI, MS[3]
1,8-Cineole (	1033	1026	55.24	RI, MS
Furfuryl ketone	1192	1180	6.45	RI, MS
α-Terpinil acetate	1351	1346	21.64	RI, MS

[1]Retention index literature from Adams [40]. [2]Calculated retention index. [3]Mass spectra from NIST02 library.

Antimicrobial activity

According to Bhavanani and Ballow [41], approximately 60% of EOs present antifungal activity, being able to be superior to the commercial synthetic antifungal products [42], and about 35% of the oils exhibit antibacterial activity. EOs of *Eucalyptus* are active against Gram-positive and Gram-negative bacteria as well as several fungal species [2]. Barbosa and coauthors [2] affirmed that *S. aureus* and *C. albicans* exhibited high sensitivity and *P. aeruginosa* most resistant to the EOs of *Eucalyptus* species.

EOs of *E. cinerea* presented values of MIC and MBC between 0.104 mg/mL to 0.416 mg/mL (Table 2). The results showed that EO of *E. cinerea* has antimicrobial activity more pronounced in the Gram-positive bacteria. Different species of *Eucalyptus*, such as *E. oleosa* F.Muell. ex Miq., *E. robusta* Sm., *E. camaldulensis* Dehnh., *E. citriodora* Hook., *E. globulus* Labill. and *E. saligna* Sm. also presented relevant activities against Gram-positive bacteria [4,43]. The resistance of Gram-negative bacteria to the action of the EO is due to the fact that its cell wall is coated by an outer membrane limiting the diffusion of hydrophobic compounds [44,45].

Silva and coauthors [46] have also investigated the antimicrobial activity of EO of different parts of *E. cinerea*, obtained during different seasons of the year, by the broth microdilution method and disk diffusion test. The results showed that the EO was active against *S. aureus*, *S. pyogenes, P. aeruginosa* and *C. albicans*, with MIC values of 0.780 mg/mL; 0.390 mg/mL; 3.120 mg/mL and 1.560 mg/mL, respectively. In the same study, the EO of *E. cinerea* showed antimicrobial activity by the disc diffusion test while the major compound 1,8-cineole showed inferior activity to EOs obtained in different seasons of the year, with MIC values between 12.50 and 50.00 mg/mL.

EOs of *Eucalyptus camaldulensis* and *E. tereticornis* Sm. showed potent antibacterial activities, inhibiting almost all of the bacteria tested, including *P. aeruginosa.* Other species in the genus, such as *Eucalyptus robusta*, *E. alba* Reinw. ex Blume, *E. citriodora*, *E. deglupta* Blume, *E. globulus* and *E. saligna*, also showed this activity [4].

Studies with EO of *E. cinerea* confirmed the antimicrobial activity of this species. However, the results found were different from those reported in the literature which can be explained by differences in the sensitivity of the strains of a given microorganism to a particular antimicrobial and differences in the concentration of active compounds in the EO. According to Hammer and coauthors [47], the oils have

hydrophobic characteristics that make it impossible to mix homogeneously with the culture medium, resulting in differences in microbial growth and different MIC values. In addition to the previously reported factors, characteristics such as plant age, seasonality, water availability, temperature, soil nutrients, altitude and UV radiation also tend to influence the chemical composition of EOs and affecting their antimicrobial properties [2,48].

Table 2. Antimicrobial activities of *Eucalyptus cinerea* essential oil

Microorganisms	MIC (µg/mL)[1]	MBC (µg/mL)[2]
Escherichia coli ATCC® 25922	0.416	0.416
Candida albicans ATCC® 10231	0.104	0.104
Staphylococcus aureus ATCC® 25923	0.208	0.208
Staphylococcus pyogenes ATCC® 12228	0.104	0.104
Pseudomonas aeruginosa ATCC® 27853	0.416	NI[3]
Chlorhexidine	1.2	1.2

[1]Minimum inhibitory concentration. [2]Minimum bactericidal concentration. [3]No inhibition.

Antioxidant activity

The antioxidant activities of EOs of *E. cinerea* are shown in Table 3. In the free radical DPPH (2,2-diphenyl-1-picryl-hydrazylhydrate) method, EO of *E. cinerea* (10-25 mg/mL) presented activity between 20.47% ± 3.18 and 7.34% ± 2.95, with statistically significant differences in relation to rutin and gallic acid standards. The low antioxidant activity found in our study was also reported in EOs of *E. radiata* A.Cunn. ex DC. by means of the DPPH assay (IC_{50} > 10,000 mg/L) [49]. However, Döll-Boscardin and coauthors [6] found a high value of IC_{50} (3209.66 µg/mL to scavenge 50% of DPPH radicals) of EO of *E. benthamii* Maiden & Cambage by the DPPH assay. However, Salem and coauthors [50] checked the best results in EO of *E. camaldulensis* subsp. *camaldulensis* and *E. gomphocephala* A.Cunn. ex DC. with values of 70 ± 3.13%, 50 ± 3.34% and 84 ± 4.64%, respectively.

In the phosphomolybdenum complex reduction assay, EO of *E. cinerea* (23.43%) presented a statistically significant difference in relation to the rutin (83.76%) and gallic acid (60.28%) standards. EO of *E. benthamii* presented a higher antioxidant activity (43.15% ± 1.08), with a statistically significant difference in relation to the ascorbic acid standard (100%) [6].

In the ABTS-radical scavenging activity, EO of *E. cinerea* presented 25.48% of antioxidant activity with statistical differences in relation to rutin (97.91%) and gallic acid (99.66%). Marzoug and coauthors [43] found a better antioxidant activity in EO of the leaves and stem of *E. oleosa* F.Muell. ex Miq. with IC_{50} values 13.0 ± 0.6 mg/L and 43.5 ± 1.4 mg/L, respectively. In the present study, medium antioxidant activity was found in the ABTS assay (IC_{50} = 484.3± 17.3 mg/L).

In general, EO of *E. cinerea* showed a low antioxidant action in the assays performed. According to Ruberto and Baratta [51], EOs with high levels of monoterpenoids are often inefficient as antioxidants. This could explain the reduced antioxidant activity of EO of *E. cinerea*, which contains monoterpenoids 1,8-cineole and α-pinene as major compounds.

Table 3. Antioxidant activities of *Eucalyptus cinerea* essential oil.

Sample	DPPH[3] AA[2] (% ± SEM)	FOSFOMOLYBDENIUM[3] AA[2] (% ± SEM)	ABTS[3] AA[2] (% ± SEM)
EO *E. cinerea*	20.47 ± 3.18[1]	23.43 ± 0.27	25.48 ± 2.62[1]
Rutin	99.50 ± 0.36	83.76 ± 0.97	97.91 ± 0.82
Gallic acid	99.93 ± 0.06	60.28 ± 0.70	99.66 ± 0.37

[1]EO (25 mg/mL). [2]antioxidant activity (AA) in percentage ± standard error of the mean. [3]statistically different results (p <0.05).

In-vitro cytotoxicity tests

MTT assay with HeLa, HRT-18, Jurkat cells

The cell viability after treatment with EO of *E. cinerea* is related to the concentrations described in Material and methods section (Figure 1). The control consists of the propyleneglycol in ethanol (1:4), which did not exceed 0.3% in culture. EO of *E. cinerea* showed no cytotoxicity up to the concentration of 1000

µg/mL against HeLa and HRT cells (Figure 1). Several species of *Eucalyptus* present cytotoxic activity as reported in EOs of *E. sideroxylon* A.Cunn. ex Woolls and *E. torquata* Luehm. against human hepatocellular carcinoma cell line (HEP-G2) and breast adenocarcinoma cell line (MCF-7) [52]. Döll-Boscardin and coauthors [5] showed that EO from the young leaves of *E. benthamii* exhibited cytotoxicity against HeLa cells at 300 µg/mL with IC_{50} of 120.57 µg/mL.

Although no cytotoxicity has been observed with EO of *E. cinerea*, the literature reports that HeLa cells are responsive to treatments performed with EOs of different species [53,54]. Likewise, EO of *E. cinerea* was not cytotoxic to HRT-18 cells. However, Murata and coauthors [55] reported antitumor activity for 1,8-cineole in different colon cancer cell lines (HCT-16 and RKO). Jurkat cells showed greater susceptibility to EO of *E. cinerea* with IC_{50} of 391.43 ± 34.57 µg/mL (Table 3). The cytotoxic effect in cells of leukemic origin (HL-60) treated with EO of *E. camaldulensis* (IC_{50} of 42.1 µg/mL) [56]. EO of *Baccharis milleflora* DC. showed cytotoxicity after 24 h of treatment, compared to Jurkat, HL-60 and Raji cells with IC_{50} values of 42.91, 23.06 and 39.15 µg/mL, respectively [57].

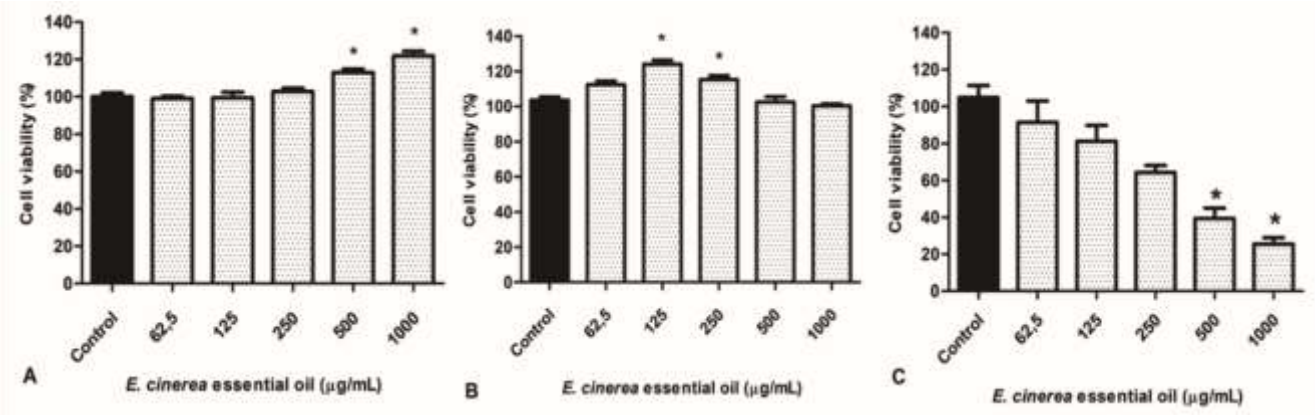


Figure 1. Cytotoxicity of EOs of *Eucalyptus cinerea* by MTT assay in relation to (A) HeLa, (B) HRT-18, (C) Jurkat cell lines after 72 h of treatment. The results are shown as mean ± SEM (n = 24). The symbol * represents a value of $p < 0.05$, which is considered significant compared to the control.

MTT assay with Calu-3 cells

Calu-3 cell viability after 72 h treatment with EOs and vapor of EO of *E. cinerea* (Figure 2). The control consisted of the propyleneglycol in ethanol (1:4), which did not exceed 0.3% in culture. The results of the cell viability experiments were expressed as IC_{50} (Table 4).

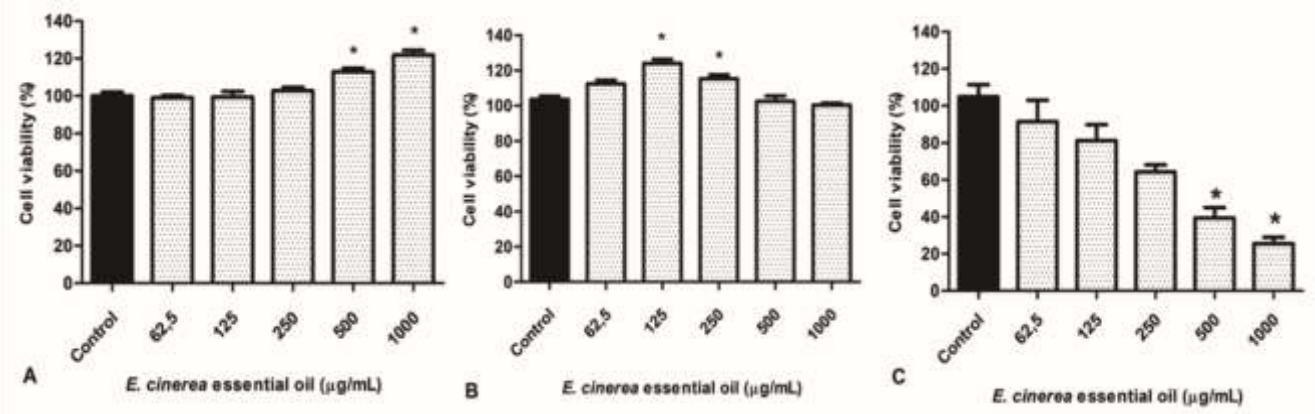


Figure 2. Cytotoxicity of *Eucalyptus cinerea* essential oil by MTT assay in relation to (A) Calu-3 cell line after 72 h of treatment with essential oil, (B) Calu-3 cell line after 72 h of treatment with essential oil vapor. The results are shown as mean ± SEM (n = 24). The symbol * represents a value of $p < 0.05$, which was considered significant compared to the control.

The EOs of *Eucalyptus* species are extensively employed in aromatherapy, especially in treating respiratory system disorders. According to Seal and coauthors [25], vaporization with EOs induces the killing of tumor cells in culture assays. However, the mechanism of transport of inhaled volatile constituents to the lungs and their effects have not yet been fully understood. In accordance with Selvarani and James [58], the

vapor phase of EO has the advantage of practicality and less toxic potential compared to the liquid phase. In this context, Calu-3 cells are advantageous in the investigation and development of inhalable drugs [59]. Reports on the biological activities of the vapor phase of EOs are still scarce. However, for the microbiological evaluation, Laird and Phillips [60] showed that vapor of EO has antibacterial effects against *E. coli, Listeria monocytogenes*, *Salmonella enteritidis, S. aureus* and *P. aeruginosa*.

According to Jakiemiu [61], monoterpenoids are more volatile than the other terpenes because they have low molecular mass, but this feature was not observed for 1,8-cineole. In the present study, essential oil vapor did not exhibit cytotoxicity in Calu-3 cells at the concentrations tested. EO of *E. cinerea* showed a significant reduction in cell viability from 500 µg/mL with an IC_{50} of 689.79 ± 29.34 µg/mL. Several studies have demonstrated the cytotoxic action of EOs in bronchoalveolar lung carcinoma cell lines. Manjamalai and coauthors [63] reported effects of the EO of *Tridax procumbens* L. in preventing lung metastasis by B16F-10 cell line in C57BL/6 mice.

Table 4. Cytotoxicity of *Eucalyptus cinerea* essential oil by MTT assay in relation to tumor cell lines after 72 h.

Cell line	IC_{50} (µg/mL) 72 h
Hela	>1000[3]
Calu-3[1]	689.79 ± 29.34
Calu-3[2]	>5000[3]
HRT-18	>1000[3]
Jurkat	391.43 ± 34.57

IC_{50}: concentration that reduces mitochondrial activity by 50%. The results are shown as mean ± SEM (n = 24). The IC_{50} values were calculated by the Least Squares method. [1]Calu- 3 cells treated with essential oil. [2]Calu-3 cells treated with essential oil vapor. [3]Estimated values according to cytotoxicity assays.

Analysis of mechanisms involved in the cytotoxic activity

Analysis of cell DNA content: Propidium iodide (PI)

The results of the proliferation of Jurkat tumor cells are shown in Figure 3. A significant reduction in DNA content was observed in 1000 µg/mL compared to the control. In the other concentrations tested, no reductions in DNA contents were detected. Pereira and coauthors [57] evaluated *B. milleflora* EO with Jurkat, Raji and HL-60 cells and reported a decrease in DNA content for all tested tumor cell lines. Döll-Boscardin and coauthors [5] reported that EO of *E. benthamii* demonstrated a statistically significant reduction in DNA content compared to vincristine used as a positive control.

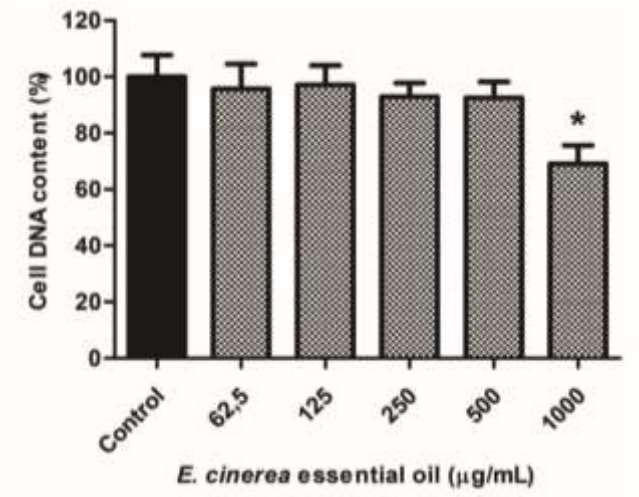


Figure 3. Determination of cell proliferation using propidium iodide by measuring the DNA content of Jurkat cells treated with *Eucalyptus cinerea* essential oil (62.5; 125; 250; 500; 1000 µg/mL) and control for 72 h. The results are shown as mean ± SEM (n =16). The symbol * represents $p < 0.05$, which was considered highly significant compared to the control (100%).

Cell cycle and DNA fragmentation assay

Table 5 displays the effect of EO of *E. cinerea* on the cell cycle progression of Jurkat cells. At 500 µg/mL, statistically significant results were detected after 72 h in Sub G0, G0/G1 and G2M phases. In the S phase,

EO did not present significant differences with the control. Besides that, no changes were observed in the cell cycle at 125 and 250 µg/mL. EO of *E. cinerea* produced an increase in the percentage of cells in the sub G0 and S phases with concomitant reduction of the cell percentage in the G0/G1 and G2/M phases. According to Döll-Boscardin and coauthors [5], changes in the cell cycle in the S, G2/M and G0/ G_1 phases revealing of cycle block/stop. EO at 500 µg/mL also induced cell fragmentation, characterized by an increase in the sub G0 population. This effect was also observed in DNA fragmentation analysis (Table 6). Grivicich and coauthors [64] affirmed that the presence of fragmented DNA is one of the alterations that precede cell death by apoptosis. To date, no studies reporting the action of *E. cinerea* EO on the cell cycle available in the literature, although numerous reports describing the action of EOs of various other species have been published.

Table 5. Distribution of Jurkat cells in cell cycle after treatment with *Eucalyptus cinerea* essential oil

Treatment	µg/mL	Cell cycle distribution (%)			
		Sub G0[1]	G0/G1	S	G2/M
Control		4.03 ± 0.30	62.01 ± 3.27	16.24 ± 2.40	18.41 ± 1.79
Essential oil	125	3.55 ± 0.31	59.59 ± 2.32	18.64 ± 1.35	14.87 ± 1.78
	250	3.87 ± 0.53	58.33 ± 1.27	18.32 ± 3.32	15.13 ± 1.34
	500	6.28 ± 1.30[2]	50.87 ± 3.48[2]	19.92 ± 1.20	12.82 ± 1.20[2]

Propylene glycol and ethyl alcohol (1:4) was used as a negative control. The results are expressed as mean ± SEM (n= 4). [1]Sub G0 represents the percentage of cells with fragmented DNA in relation to the total events (30.000) analyzed. [2]Statistical differences (p<0.05) when one-way ANOVA followed by Tukey's post-hoc test was applied in relation to the control during 72 h of the treatment.

The percentage of DNA fragmentation along with cell cycle analysis was performed for Jurkat cells (Table 6). As described in the cell cycle assay, EO of *E. cinerea* at 500 µg/mL caused DNA fragmentation with a statistically significant difference to the control. Considering the results, *E. cinerea* EO probably lead to a death mechanism associated with apoptosis. Wong [65] opines that apoptosis is a superior mechanism for feasible therapeutic interventions on the pathophysiology of cancer.

Considering the mechanism of action, several studies have been reported that EOs inducing cell death [66,67]. Apoptosis death is a process also observed for several antitumor agents [64,68]. Eugenol, a common volatile compound of several EOs, displayed apoptosis in HL-60 cells, including DNA fragmentation and formation of DNA ladders in agarose gel electrophoresis.

Justus and coauthors [68] analyzed *Lavandula dentata* L. EO on Calu-3 lung cancer cells in both vapor and liquid phases and reported a significant reduction of cell viability by reaching 84% cytotoxicity in vapor phase, showing a time-dependent profile. Although both necrosis and apoptosis mechanisms were involved in Calu-3 cell death, necrosis seemed to be the dominant cell death pathway.

Table 6. DNA fragmentation after treatment with *Eucalyptus cinerea* essential oil.

Treatment	Fragmentation (%)
Control	16.39 ± 2.14
125 µg/mL	18.60 ± 1.09
250 µg/mL	19.46 ± 3.80
500 µg/mL	29.73 ± 2.17[1]

Results are expressed as mean and standard deviation (SD) of n = 4 with analysis of 30,000 events per sample. [1]Statistical differences (p <0.05) when one-way ANOVA followed by Tukey's post-hoc test was applied in relation to the control during 72 h of the treatment.

Anatomical analysis

The leaves of *Eucalyptus cinerea* (Figure 4 A, B), in frontal view, show epidermal cells with straight and thin anticlinal walls on both sides (Figure 4 C, D). The leaves are amphistomatic, and two types of stomata, anomocytic and actinocytic, are observed (Figure 4 C, D). The stomata measure an average of 23 µm in length. Both amphistomatic [16,70] and hypostomatic [70] leaves were met in the genus, while amphistomatic

leaves were more frequent. Anomocytic stomata are typical in *Eucalyptus* [16,69,71] whereas, actinocytic stomata were commonly found in other genera of Myrtaceae [72].

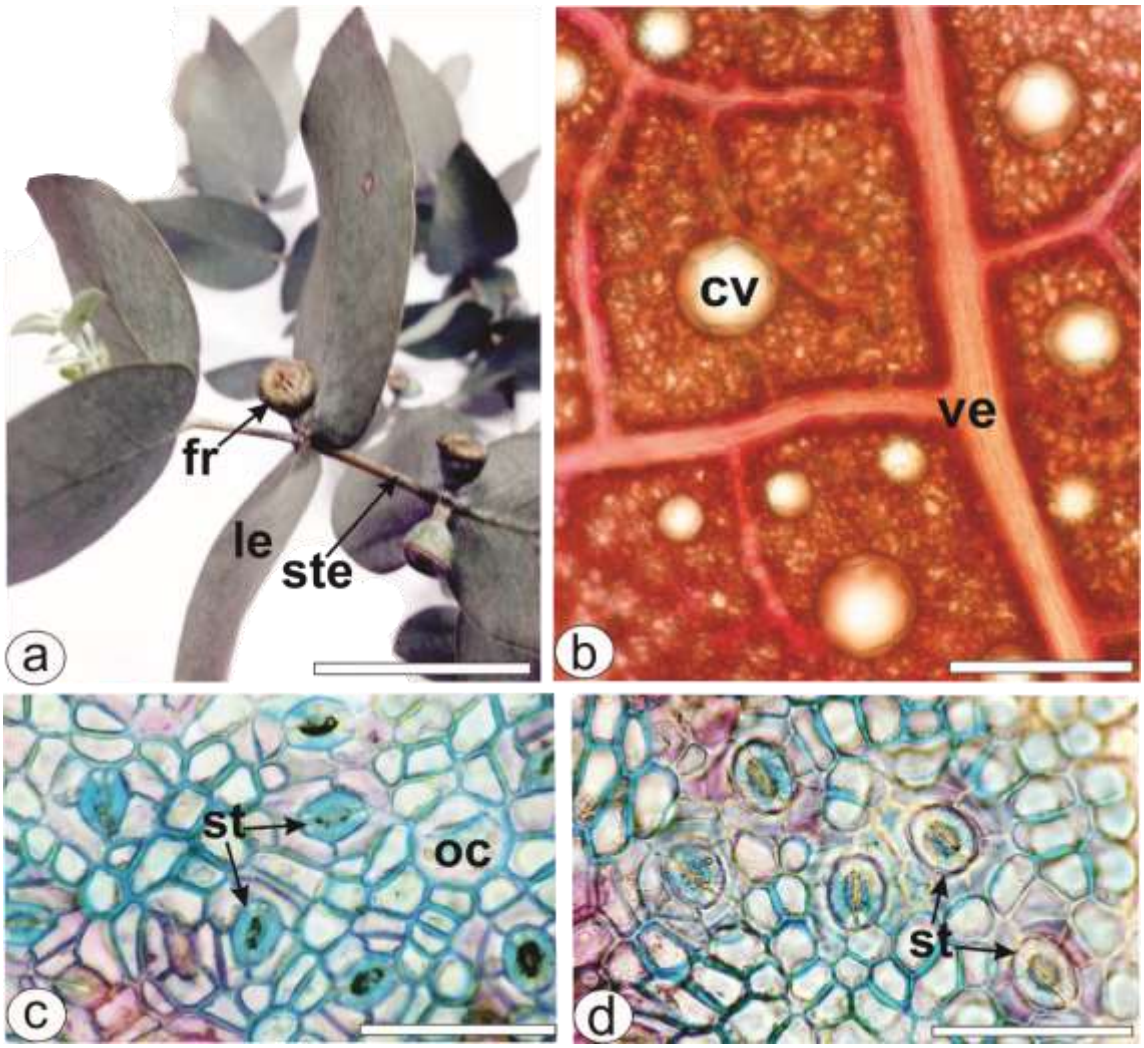


Figure 4. Morpho-anatomy of *Eucalyptus cinerea*. [Light microscopy; stained in safranin (b) and Astra blue (c, d)]. a. Photo of twigs with fruits. b and d. Lower epidermis. c. Upper epidermis. [cv- secretory cavities, fr- fruits, le- leaves, oc- overlying cells, st- stomata, ste- stem, ve- veins. Scale bar: a = 5 cm; b = 200 µm; c, d = 50 µm.

E. cinerea leaf shows a pair of overlying cells of secretory cavities at the same level as the stomata and other epidermal cells (Figure 4 C). These cells are also found in many other species of *Eucalyptus* [69,71]. Although the overlying cells are frequent in the genus, variations in the number of cells can be found; for example, the overlying cells are made up of up to four cells in *E. pyrocarpa* L.A.S.Johnson & Blaxell [71].

The leaves of *E. cinerea*, in cross-section, show uniseriate epidermis with smooth and thick cuticle and papillae (Figure 5 A, B). Thick cuticle and papillae were also observed in many species of *Eucalyptus* [16,69,71]. However, papillae were not found in *E. platypus* Hook.f., *E. spathulata* Hook. and *E. viridis* F.Muell. ex R.T.Baker [72].

The mesophyll is isobilateral and is formed by about three layers of palisade parenchyma on both sides and two layers of spongy parenchyma. Small bicollateral vascular bundles surrounded by parenchymatous sheath are immersed in the mesophyll (Figure 5 A). Druses and prismatic crystals are found in the mesophyll. Isobilateral mesophyll is common in *Eucalyptus* [16,69]. However, a dorsiventral organization can be met in *E. globulus* subsp. *bicostata* (Maiden, Blakely & Simmonds) J.B.Kirkp. [70], *E. grandis* W.Hill, *E. pilularis* Sm., and *E. resinifera* Sm. [71].

In the present study, secretory cavities (Figure 4 B) are observed in the mesophyll, especially in the sub-epidermal region on both sides of the leaves (Figure 5 B). Secretory cavities are common in the genus [16,69,71]. In Myrtaceae, they are usually found in the palisade parenchyma, typically in contact with the adaxial epidermis, but sometimes they can occur in both adaxial and abaxial palisades [72].

The midrib, in cross-section, is slightly convex on both sides (Figure 5 C). This shape was observed in *E. pyrocarpa* [71], *E. badjensis* Beuzev. & Welch and *E. benthamii* Maiden & Cambage [69]. Flat-convex shape has been described in several species of *Eucalyptus*, such as *E. grandis*, *E. resinifera*, *E. saligna* and *E. urophylla* S.T.Blake [69,71]. Migacz and coauthors [69] reported that the midrib shape and the vascularization pattern may help in the differentiation of *Eucalyptus* species.

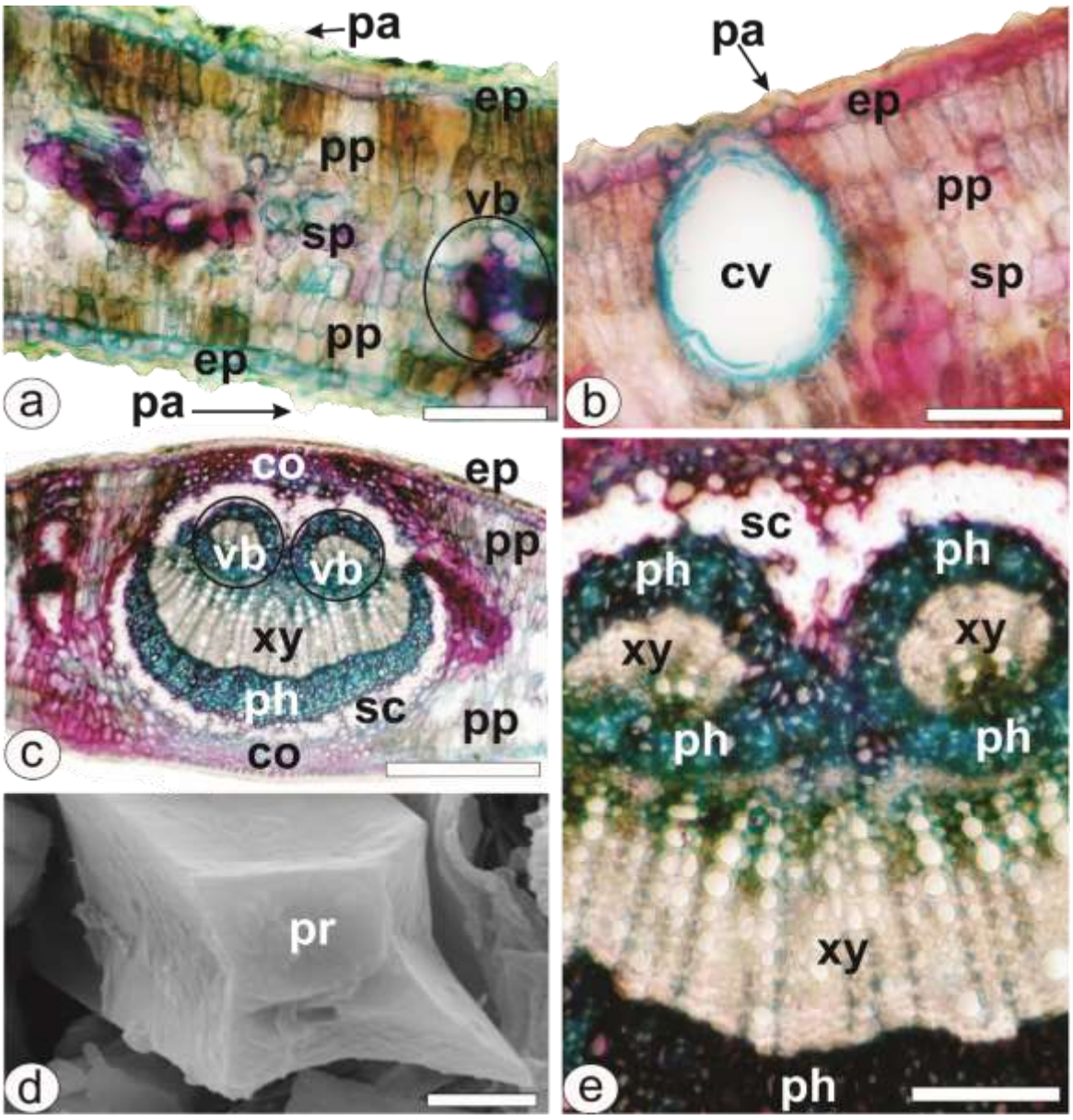


Figure 5. Leaf anatomy of *Eucalyptus cinerea*. [Light microscopy: a, b, c, e; FESEM: d]. a-c, e: Transverse sections (a, b: mesophyll; c, e: midrib) – stained in Astra blue/basic fuchsin. d: View of a prismatic crystal. [co- collenchyma, cv- secretory cavity, ep- epidermis, pa- papillae, ph- phloem, pp- palisade parenchyma, pr- prismatic crystal, sc- sclerenchymatous sheath, sp- spongy parenchyma, vb- vascular bundle, xy- xylem. Scale bars: a, b, d, e = 50 µm; c = 200 µm.

The unilayered epidermis is covered by a thick and striated cuticle. The sub-epidermal collenchyma appeared as about 4 layers. Crystals were spread in the midrib as previously described for the mesophyll (Figure 5 D). The midrib stele consists of a large bicollateral vascular bundle in an open arc and two dorsal trace types. A similar arrangement was also found in *E. benthamii*, *E. dunnii* and *E. globulus* [69]. However, different patterns were observed in other species of *Eucalyptus*, such as collateral vascular bundle in an open arc with one dorsal plate in *E. grandis* [69] and a collateral vascular bundle in an open arc with invaginated ends in *E. saligna* [16]. The perivascular fiber cap adjoining the phloem (Figure 5 C, E) was also observed in *E. badjensis*, *E. benthamii* and *E. dunnii* [69]. Migacz and coauthors [69] reported that this feature could help in the identification of *Eucalyptus* species.

The young stem is circular in cross-section (Figure 6 A). The epidermis is uniseriate and covered by a thick and striated cuticle. Different stem shapes can be found in *Eucalyptus,* such as rectangular in *E. grandis*, circular in *E. urophylla* [74] and *E. saligna* [16] and stellate near the shoot apex and rectangular in developed stem in *E. grandis* [75]. Beneath the epidermis, cortical parenchyma has about 16 layers and there are some secretory cavities (Figure 6 A, B), measuring 110 µm in diameter on average. Secretory cavities in the stems of *E. grandis*, *E. urophylla* and *E. saligna* measure 78, 45 and 40-110 µm in diameter, respectively [16,74]. The vascular cylinders in > 2 mm wide stems present cambia forming phloem outward and xylem inward. Sclerenchymatous cells are seen along the periphery of the vascular system (Figure 6 A, C). This characteristic is also observed in *E. pilularis*, *E. tetrodonta* F.Muell. and *E. nitens* (H.Deane & Maiden) Maiden [75].

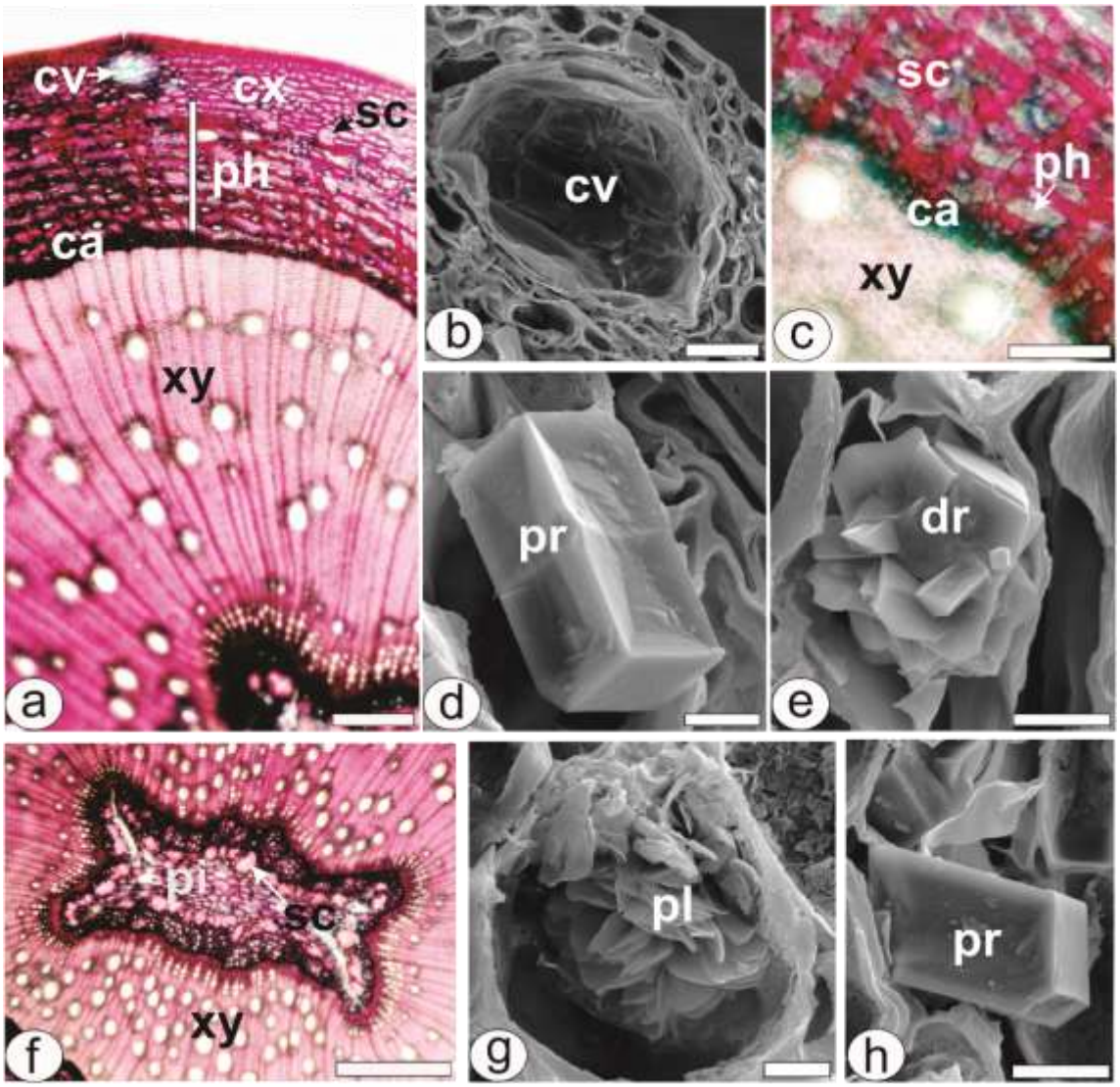


Figure 6. Stem anatomy of *Eucalyptus cinerea*. [Light microscopy stained in Astra blue/basic fuchsin: a, c, f; FESEM: b, d, e, g, h]. Transverse sections of stem (a- c, f), and views of crystals (d, e, g, h). [ca- cambia, cx- cortex, co- collenchyma, cv- secretory cavity, dr- a blocky and tabular crystal druse, ep- epidermis, ph- phloem, pi- pith, pl- platy aggregate cluster, pp- palisade parenchyma, pr- prismatic crystal, sc- sclerenchymatous sheath, xy- xylem. Scale bars: a, f = 100 µm, c = 50 µm, b = 20 µm, h = 10 µm, d, e, g = 5 µm.

The pith is arranged in a stellate shape in cross-section and is formed by parenchyma cells, thick-walled fibers, and crystal idioblasts (Figure 6 F). Pith with different shapes was found in other species, such as rectangular in *E. saligna* [16], *E. microcorys* F.Muell., *E. marginata* Donn ex Sm. and *E. grandis* [75]. The shape of the vascular tissue can vary, depending on the age of the stem. For example, near the fourth node, *E. camaldulensis*, *E. globulus* and *E. nitens* showed rectangular or slightly stellate, whereas *E. tetrodonta* and *E. pilularis* presented rectangular or circular shapes.

In the present study, *E. cinerea* showed different morphotypes of crystals in the mesophyll, midrib and stem; these are prisms (Figure 6 D, H), platy aggregate clusters (Figure 6 G), and blocky and tabular crystal

druses (Figure 6 E). Several species detoxify soluble oxalic acid as insoluble calcium salts in their tissues. They can be prismatic crystals, druses, raphides, styloids or crystal sand and their shapes are important for diagnostic purposes [28,76,77,78]. Weiner and Dove [79] have reported that the excess of calcium is usually precipitated in calcium salts such as oxalate, carbonate, phosphate, silicate, sulfate, citrate and malate.

The three types of crystals were analyzed by EDS for their elemental composition and their spectra showed prominent peaks for calcium. The prisms in the midrib showed large peaks of calcium (24.29%), carbon (56.75%), and oxygen (18.97%) (Figure 7 A). The blocky and tabular druses evidenced large calcium (50.00%), carbon (19.80%), and oxygen (30.21%) peaks (Figure 7 B). The platy aggregation cluster showed large calcium (28.06%), carbon (38.48%), oxygen (29.48%), and magnesium (3.98) peaks (Figure 7 C). The prisms in the stem indicated large calcium (26.72%) and oxygen (73.28%) peaks (Figure 7 D).

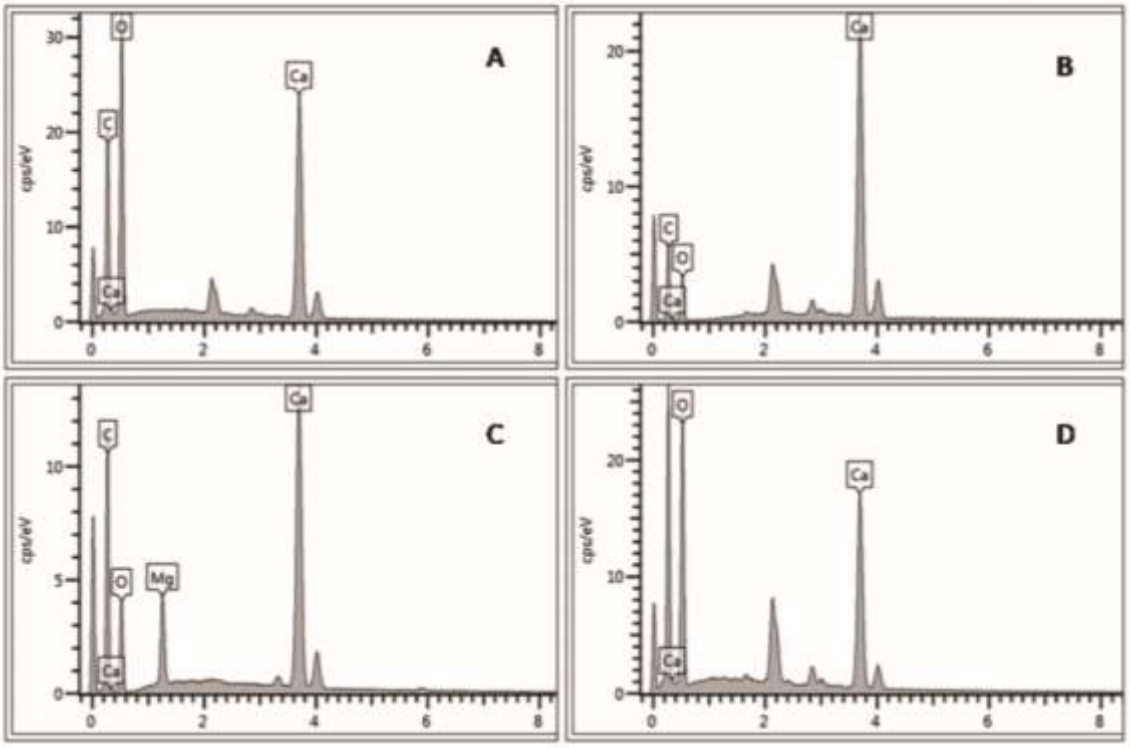


Figure 7. EDS (energy-dispersive X-ray spectroscopy) spectra of crystals of *Eucalyptus cinerea*. A. Prismatic crystal in the midrib; B. Blocky and tabular crystal druse; C. Platy aggregate cluster; D. Prismatic crystal in the stem. The major unlabeled peaks in these spectra represent gold (Au), which was used to coat the samples.

Histochemical Analysis

The histochemical tests show the presence of lipophilic compounds in the cuticle in the leaf blade (Figure 8 A), midrib (Figure 8 D) and stem. The secretory cavities also present volatile oils that reacted with Sudan III (Figure 8 D). Phenolic compounds reacted positively with ferric chloride and are found in the mesophyll (Figure 8 B) and midrib phloem (Figure 8 C) in the leaves, cortex, phloem (Figure 8 F) and xylem parenchyma in the stem (Figure 8 E). Small rounded starch grains are observed in the pith. Lignin is found in the vessels, tracheids and sclerenchyma cells present in the leaf midrib, stem cortex and pith.

Migacz and coauthors [69] observed phenolic compounds in the phloem in six species of *Eucalyptus*, but in higher amounts in *E. grandis* and *E. saligna*. Several idioblasts containing tannin were seen in the cortex in *E. urophylla* [74]. *Eucalyptus saligna* showed several calcium oxalate prisms, secretory cavities and phenolic idioblasts in the stem [16].

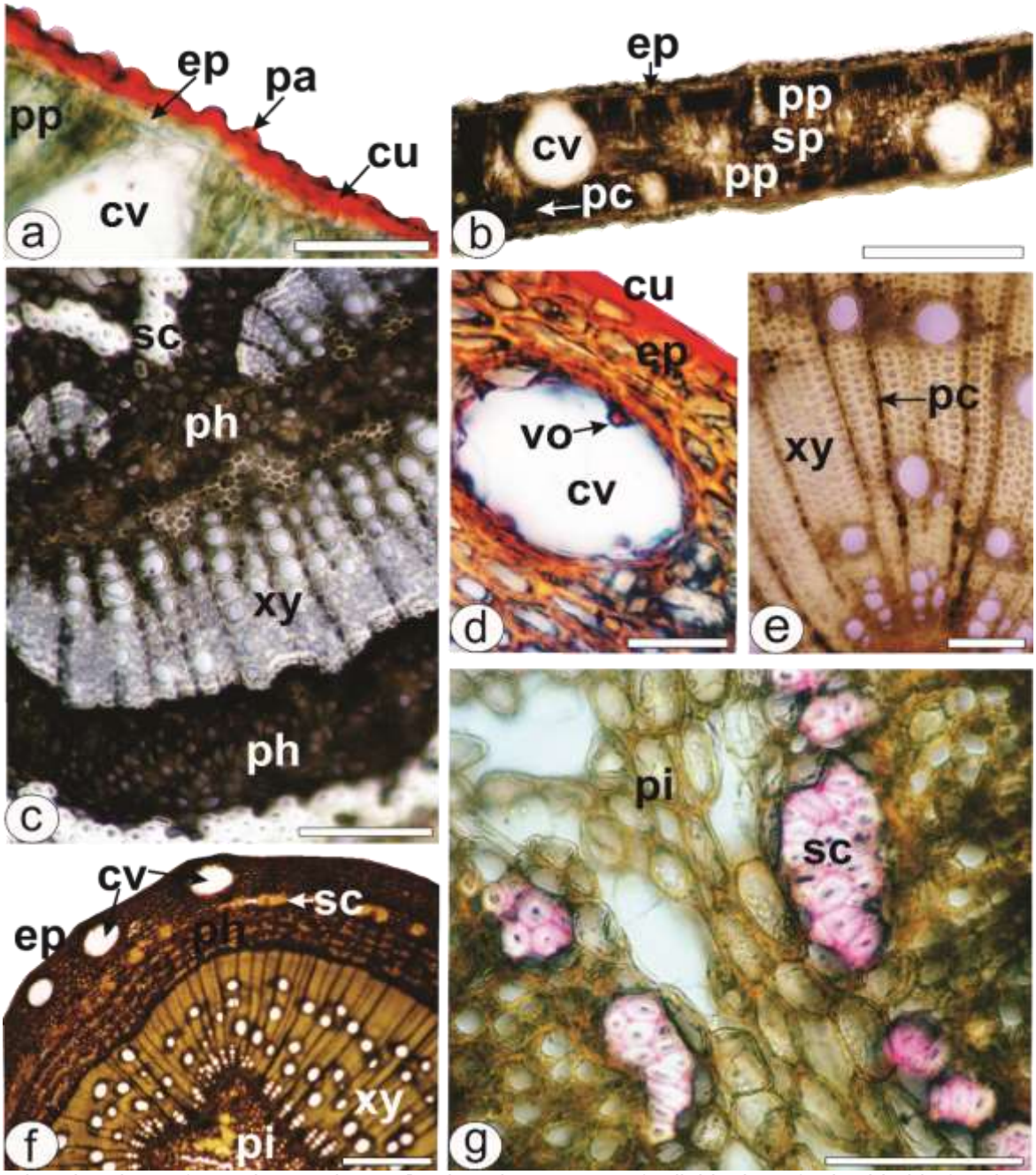


Figure 8. Histochemistry of *Eucalyptus cinerea* [a, d: sudan III to test for lipids; b, c: ferric chloride for phenolics; e, f: potassium dichromate solution (10%) for phenolic compounds; g: phloroglucinol/HCl for lignin. Transverse sections – a-c: leaf; d-g: stem [co: collenchyma, cx- cortex, cu: cuticle, cv: secretory cavities, en: endodermis, eo: essential oil, ep: epidermis, gp: ground parenchyma, pc: phenolic compounds, ph: phloem, pi: pith, pr: prismatic crystal, sc: sclerenchyma, sg: starch grains, sp- spongy parenchyma, pp- palisade parenchyma, vo- volatile oil, xy: xylem]. Scale bar: b, f = 200µm; a, c, e, g = 50µm; d = 20 µm.

CONCLUSION

The present study has demonstrated the antioxidant, antimicrobial and cytotoxic activities. It also explores the chemical composition of EOs and anatomical characters of the leaves and stems of *Eucalyptus cinerea* cultivated in Brazil. The main constituent of the EO was 1,8-cineole (55.24%). The EO presented low antioxidant activity in the three methods tested. In the microbiological test, the best results were against *C. albicans* and *S. pyogenes* with MIC of 0.104 mg/mL. The EO reduced the viability of the Jurkat cells (IC_{50} 391.43 µg/mL) and Calu-3 cells (IC_{50} 689.79 µg/mL) by the MTT assay, decreased DNA content in the Jurkat cells after 72 h of treatment and finally increased the percentage of cells in the sub G0 and S phases with concomitant reduction of the cell percentage in the G0/G1 and G2/M phases and caused DNA fragmentation of 29.73%. These results indicate that the EO has possibly lead to a death mechanism associated with apoptosis.

The anatomical features that could help in the identification *E. cinerea* include: isobilateral and amphistomatic leaves; epidermis with papillae, anomocytic and actinocytic stomata; slightly biconvex midrib with one large bicollateral vascular bundle in open arc and two smaller dorsal bundles; circular stems; sclerenchyma in the cortex and pith; and prisms, platy aggregate clusters and blocky and tabular crystal druses in the mesophyll, midrib and stem. Histochemical tests revealed the presence of lipophilic and phenolic compounds, lignified elements and starch grains.

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