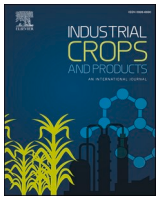
Industrial Crops & Products 186 (2022) 115180

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Untargeted metabolomics used to describe the chemical composition and antimicrobial effects of the essential oil from the leaves of *Guatteria citriodora* Ducke

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ARTICLE INFO

Keywords:
Citronellal
Citronellol
Inhibitory concentration
Natural products
Membrane integrity
Volatile oils

ABSTRACT

Plant oils are sources of metabolites that have enormous potential for industrial applications. Herein, the chemical profile and in vitro antimicrobial activity of the essential oil (EO) from the leaves of *Guatteria citriodora* Ducke (Annonaceae) have been investigated for the first time. The composition of the hydrodistilled EO was analyzed using gas chromatography-mass spectrometry (GC-MS), which permitted the identification of oxygenated monoterpenes as the most highly representative class, and included citronellal (40.99%) and citronellol (14.6%) as the main compounds. The antimicrobial activity of *G. citriodora* EO (GcEO) was evaluated against pathogenic bacteria and phytopathogenic fungi. The experimental design was completely randomized (CRD), and used doses for each microorganism. Gram-positive strains were the most sensitive with a minimum inhibitory concentration (MIC) of 5.0 μL mL^{-1}, while Gram-negative strains were 10.0 μL mL^{-1}. The most potent antifungal activity was against *Alternaria alternata* (MIC of 1.25 μL mL^{-1}). In addition, it fully inhibited *A. alternata* conidia germination at the minimum inhibitory concentration. The nucleic acid and soluble protein contents were significantly released from the conidia of *A. alternata* after treatment with GcEO. Using SEM (scanning electron microscopy), morphological alterations were observed in the conidia, which indicates that a lesion in the cytoplasmic membrane is one of its mechanisms of action. Overall, these results indicate that GcEO is an antimicrobial agent with potential applications in the agriculture, food, and pharmaceutical industries.

1. Introduction

The Amazon region has great prominence due to it housing the largest area of rainforest in the world. In this ecosystem, about 11% of the world's tree species are found (Cardoso et al., 2017), and it is estimated that there are around 50 000 vascular plant species (Hubbell et al., 2008). A recent study on the number of trees on Earth indicates the existence of ~73 000 tree species (Gatti et al., 2022), while the survey of the number of trees in the Amazon region suggests ~16 000 Amazonian tree species (Ter Steege et al., 2016). Given this scenario, the rational and sustainable use of tropical forest products (e.g., leaves, nuts, seeds, bark, resins, and oils) can potentially contribute to the preservation of forests and resources, in addition to promoting a source of income for the native population, which is especially important for poor rural communities (Nascimento et al., 2019; Mello et al., 2020). Therefore, studies have been conducted to evaluate the active ingredients found in

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https://doi.org/10.1016/j.indcrop.2022.115180
Received 13 January 2022; Received in revised form 1 May 2022; Accepted 2 June 2022
Available online 18 June 2022

Amazon biodiversity, as well as their use in pharmacology, cosmetics, and agriculture. Among the forest products, essential oils (EOs) have aroused great scientific interest since they encompass volatile molecules, which have aromatic features of biotechnological and industrial interest (Ricardo et al., 2017; Silva et al., 2018; Souza et al., 2020).

EOs are characterized as complex mixtures of volatile substances, which are generally lipophilic and water-insoluble, and are mainly produced in flowers and leaves, but are also present in stems and bark, though to a lesser extent in seeds and roots (Morone-Fortunato et al., 2010; Calvo-Irabien, 2018). Molecules of EOs are biosynthesized, accumulated and secreted in specialized anatomical structures such as secretory idioblasts, canals, cavities/ducts, or glandular trichomes (Pickard, 2008; Tiwari, 2016). The majority of compounds normally found in Eos, mainly originate from three biosynthetic pathways, (1) the plastidal 2-*C*-methylerythritol-4-phosphate (MEP) pathway, which leads to mono- and diterpenes, (2) the mevalonic acid (MVA) pathway, which acts in the cytosol producing sesquiterpenes, and (3) the shikimate pathway that leads to benzenoid derivatives (Bergman and Phillips, 2020; Rehman et al., 2016).

The biological properties of EOs have been known for a long time. Previous reports have shown the biotechnological potential of EOs, which is due their wide-spectrum of biological activities, and the fact that they are eco-friendly (Issa et al., 2020; Xiang et al., 2020; Yilmaz, 2020). Several biological activities of EOs, such as insecticidal, antiparasitic, antiviral, antibacterial and antifungal activity (Battisti et al., 2021; Sobrinho et al., 2021; Vega Gomez et al., 2021), have been shown in the literature. *In vitro* studies have shown that essential oils are active against bacteria and fungi and act mainly by disrupting cell membrane integrity by inducing an increase in the permeability of the membrane and the leakage of genetic material (Al-Shuneigat et al., 2020; Silva et al., 2019; Xu et al., 2018; Zhang et al., 2020).

Various EOs from plants that are endemic to the Amazon, including species of Burseraceae, Lauraceae, Cyperaceae, Piperaceae and Annonaceae (Maia and Andrade, 2009) have already been studied. *Guatteria citriodora* (Annonaceae), popularly known as 'laranjinha', is distributed in the Amazon Rainforest, and is mainly found in Brazil, Bolivia, Colombia, Ecuador, Peru, Suriname and Venezuela (GBIF, 2019). There are few reports of the popular use of this plant; however, communities in the interior of the Amazon often use its leaves to make a relaxing tea. Previous phytochemical investigation of this species has described it as having a rich isoquinoline alkaloid content with antiplasmodial and antibacterial activities (Rabelo et al., 2014) but, despite this, there are no published data on the chemical composition of the EO of *G. citriodora*. Nevertheless, the phytochemical composition of EOs of some species belonging to the genus *Guatteria* has revealed certain bioactive properties. *Guatteria* EOs have shown a predominance of oxygenated sesquiterpenoids with biological activities associated to anticancer (Branches et al., 2019; Costa et al., 2020), antileishmanial (Siqueira et al., 2015) and antimicrobial (Alcântara et al., 2017) properties.

Another important aspect, highlighted here, is how the selected molecule acts on a membrane-active mechanism to strengthen its effects against microorganisms. In this study, the results obtained are discussed following the current context of our knowledge regarding the effects of essential oils in relation to morphological alterations to fungal cell structures, as published in Pimentel et al. (2018) and Souza et al. (2020). Herein, it was hypothesized that GcEO induces the leakage of cellular components of microorganisms (fungi), which may lead to structural and/or functional alterations. As such, the aim of this study was to investigate the chemical composition and the antimicrobial potential of essential oil from leaves of *G. citriodora*, as well as identify the possible antifungal mechanism associated with the morphophysiological alterations.

2. Materials and methods

2.1. Information on the origin of the material

The leaves from thirty matrices of *G. citriodora* were randomly collected in 2019 and 2020 from the Adolpho Ducke Forest Reserve (2° 48′ 72″ S, 59° 53′ 32″ W), Manaus, Amazonas, Brazil. Authentication of the plant species was carried out by INPA taxonomists via comparison with the original voucher (No. 14 570) deposited in the INPA Herbarium, in Manaus. The collection was performed during the morning (8 am), in the month of March (mean rainfall $\cong$ 300 mm month^{-1}). The climate of Manaus is classified as type Af, hot and humid according to Köppen, with an annual average rainfall of 2420 mm and an annual mean temperature of 26.7 °C (Alvares et al., 2013).

2.2. Extraction of the essential oil

Leaves were air-dried (300 g) at room temperature (25 ± 2 °C) for 7 days and then finely ground and subjected to hydrodistillation during 3 h using a Clevenger-type apparatus. Water was removed from the EO by drying over anhydrous sodium sulfate, then kept in sealed amber vials and stored at 4 °C (Farahbakhsh et al., 2021) until GC−MS analysis (24 h later) and biological assays (72 h later). The essential oil yield was estimated on a dry weight basis as 1.74% (v/w).

2.3. Essential oil analysis

The analysis of the GcEO was performed on a gas chromatograph-mass spectrometer (Shimadzu QP2010 Ultra GC–MS, Kyoto, Japan) equipped with a HP-5MS capillary column (30 m x 0.25 mm; 0.25-μm film thickness). The GC–MS parameters were set as follows: injector temperature, 220 °C; column temperature, 60–240 °C at a rate of 3 °C min^{-1}; detector temperature, 250 °C; helium used as the carrier gas at a constant flow rate of 1 mL min^{-1}; ionization energy 70 eV; mass scan range of *m/z* 30–500. The injection was performed with an aliquot of 1.0 μL of GcEO (0.5 mg mL^{-1} in ethyl acetate) and was injected in the splitless mode. For the relative amount of each constituent of the EO, the normalized peak area was used to express the relative percentage of the oil constituents. Identification of chemical compounds was achieved by matching their mass spectra data to the NIST17 mass spectral library, with matches above 98% similarity together with the manual annotation of the fragments present in the mass spectra. Furthermore, the confirmation of the identification was performed via the calculation of the retention indexes (RI) according to the Van den Dool and Kratz equation (Van den Dool and Kratz, 1963) in comparison with a homologous series of linear hydrocarbons (C7-C30), and those reported in the literature (Adams, 2007; Babushok et al., 2011).

2.4. Microorganisms

The following microorganisms were used in the assays: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25 923), *Klebsiella pneumoniae* (ATCC 10 031), and *Salmonella enterica* (ATCC 10 708). These strains were donated by the laboratory of plant toxins at the Department of Biochemistry and Molecular Biology of the Federal University of Ceará (UFC), where the original cultures are maintained.

The plant pathogenic fungi, *Alternaria alternata* (INPA 2617), *Aspergillus flavus* (INPA 3687), *Fusarium oxysporum* (INPA 2752) and *Colletotrichum guaranicola* (INPA 1343), came from the Microbiological Collection at the National Institute for Amazonian Research (MCTI-INPA), Amazonas, Brazil.

Both sets of species were cultivated following the instructions of the suppliers.

2.5. Antibacterial and antifungal assays

The antibacterial tests on *B. subtilis*, *S. aureus*, *K. pneumoniae* and *S. enterica* were performed in 96-well microtiter plates. Aliquots of 100 μL of Mueller Hinton broth containing the bacterial cells (approximately 5 $\times 10^5$ CFU/mL) were incubated in the dark, at 37 °C, with serially diluted GcEO (dissolved in 0.1% (v/v) Tween-80) at final concentrations of 0.312–40 μL mL^{-1}, equivalent to 296 – 38 000 ppm. Negative and positive controls for growth inhibition were composed of 0.1% (v/v) Tween-80 and stock solutions of 1 mg mL^{-1} norfloxacin, respectively. Bacterial growth was investigated by means of spectrophotometric readings with absorbance at 630 nm (A_{630}) in an automated microplate reader (Epoch, BioTek Instruments, Inc., USA).

Fungi cultivated for 14 days were the source of the conidia. The suspensions were obtained and adjusted to 2×10^5 conidia mL^{-1}. Subsequently, 10 μL of conidial suspension was incubated with 90 μL of yeast potato dextrose broth in the microtiter plate, for 16 h at 26 ± 2 °C. GcEO (100 μL) was added in serial dilution (0.312–40 μL mL^{-1} final concentrations, equivalent to 296 – 38 000 ppm) to each well and the plate was incubated at 26 ± 2 °C for 48 h. Negative and positive assays for growth inhibition consisted of 0.1% (v/v) Tween-80 and stock solutions of 2 mg mL^{-1} mancozeb, respectively. The A_{630} was measured on an automated microplate reader (Elx800, Biotek) to observe antifungal activity.

2.6. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal or fungicidal concentration (MBC/MFC)

2,3–5-triphenyl tetrazolium chloride (TTC, 1%) solution (10 μL), which indicates the activity of dehydrogenase enzymes involved in the process of cellular respiration, was added to each well of the microtiter plate, which was then incubated at 37 °C for 1 h. The MIC was defined as the lowest concentration showing no color change (clear). The MBC/MFC were determined by subculturing of 5 μL cultures on Mueller Hinton or potato dextrose agar at 26 °C for up to 48 h. The plates without any visible growth were marked as MBC/MFC.

2.7. Determination of the 50% inhibitory concentration (IC_{50}) and curve fitting

The bacterial and fungi inhibition percentage was calculated using Eq. 1:

$$\%\ inhibition = 100 - \left[\frac{100 \times (A_{630}\ of\ treated\ well - Average\ of\ background\ A_{630})}{(A_{630}\ of\ growth\ well - Average\ of\ background\ A_{630})}\right] \quad (1)$$

The modeling of the percentage of inhibition and IC_{50} was fitted using the model of nonlinear regression proposed by Rautenbach et al. (2006) using Eq. 2:

$$Y = \frac{bottom + (top - bottom)}{1 + 10^{[log(IC_{50} - x) \times hill\ slope]}} \quad (2)$$

where top is the Y-value at the top plateau (inhibition at high GcEO concentrations); bottom is the Y-value at the bottom plateau (response when GcEO is absent); hill slope is the slope of the curve, x represents the logarithm (10-base) of [GcEO]. The inhibition parameters were calculated using the GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA).

2.8. Conidial cultivation assay

Conidia from *Alternaria alternata* were used to determine the effect of GcEO on conidial germination and antifungal mechanisms. In sterile glass depression slides, 5 μL of *A. alternata* conidia suspension (2×10^5 conidia mL^{-1}) were incubated with 5 μL of GcEO at 0.625, 1.25 and 2.5 μL mL^{-1} final concentration, corresponding to 1/2 ×MIC, MIC and 2 ×MIC values. The depression slides were placed in Petri dishes containing wet filter paper at 26 °C ± 2 °C and maintained in the dark for 16 h. Afterwards, the slides from each set were studied under a microscope (Zeiss AxioLab A1). In the reference control, equal amounts of 0.1% Tween-80 (v/v) were used as the negative reference, and the conidia were scored as germinated if the germ tube length was equal or superior to the length of the conidia. At least 50 conidia within each replicate were observed. The germination inhibition percentage was calculated according to the following formula:

$$\%\text{germination inhibition} = \left[\frac{(Gc - Gt)}{Gc}\right] \times 100$$

where Gc is the number of germinated conidia in negative control slides; Gt represents the number of germinated conidia in GcEO-treated slides. Three independent experiments were performed.

2.9. Determination of release of cell constituents

The leakage of cytoplasmic contents from *Alternaria alternata* was determined according to the method of Ma et al. (2018) with minor modifications. An aliquot of GcEO at 0.625, 1.25 and 2.5 μL mL^{-1} final concentrations (in 0.1% Tween-80) was mixed with 2 mL of conidial suspensions (5×10^7 conidia mL^{-1}) prepared in 10 mM phosphate buffered saline (pH 7.4). The assays were conserved at 26 °C ± 2 °C for 16 h. After incubation, the samples were centrifuged at 4000g for 10 min at 4 °C. Aqueous phases were used for determining nucleic acid and protein contents after 0.22 μm filtration. Aliquots of 0.1% Tween-80 (v/v) was used as the negative control. Leakage of nucleic acids was measured by detecting absorbance at 260 nm (A_{260}). Bradford reagent with bovine serum albumin was used as the standard to quantify the release of protein (Bradford, 1976). Each experiment was performed in triplicate.

2.10. Analysis of cellular morphology using scanning electron microscopy (SEM)

An aliquot of 100 μL of *A. alternata* conidia suspension (2.5×10^5 conidia mL^{-1}) was incubated with 100 μL of GcEO (1.25 μL mL^{-1} final concentration, corresponding to MIC value) at 26 °C ± 2 °C for 16 h. Then, the conidia were recovered by centrifugation (5000g, 22 °C) for 10 min and washed twice with PBS buffer. The conidia were fixed in glutaraldehyde at 2.5% in 0.05 M phosphate buffer, pH 7.4 for 24 h. Next, they were washed in the same buffer, post-fixed in 2% osmium tetroxide and dehydrated by immersing the material in an increasing stepwise series of ethyl alcohol (30% up to 100%). Next, the samples were critical-point-dried with CO_2 and coated with gold for examination using SEM (LEO, 435 VP) operating at 20 kV at 1000 × magnification.

2.11. Statistical analyses

The experimental design was a completely randomized (CRD), and

used the EO doses (independent variable) shown in Fig. 3 for each microorganism (% germination inhibitor, dependent variable) and Fig. 4 (effects of GcEO on leakages of specific cell molecules, dependent variable). All analyses of the samples were carried out in triplicate (with $n = 3$ or 4) and all results are expressed as mean estimative ± standard deviation and compared using an analysis of variance (ANOVA) followed by Tukey's post hoc tests using Graphpad Prism 8.0 software (Graphpad Software, Inc.).

3. Results and discussion

3.1. Chemical analysis

Untargeted metabolomic investigation is the description of the

Table 1
Chemical composition of essential oil from *Guatteria citriodora* leaves extracted using the hydrodistillation method.

Components[a]	RI HP-5MS	RI Lit.	Molecular formula	CAS number	Relative area (%)	Identification
α-Pinene	936	939	$C_{10}H_{16}$	80–56–8	0.36 ± 0.02	RI, MS, BI
β-Pinene	980	979	$C_{10}H_{16}$	127–91–3	0.97 ± 0.06	RI, MS, BI
Sulcatone	990	985	$C_8H_{14}O$	110–93–0	0.05 ± 0.00	RI, MS, BI
Myrcene	993	990	$C_{10}H_{16}$	123–35–3	0.36 ± 0.02	RI, MS, BI
α-Phellandrene	1007	1002	$C_{10}H_{16}$	99–83–2	0.16 ± 0.01	RI, MS, BI
o-Cymene	1026	1026	$C_{10}H_{14}$	527–84–4	0.31 ± 0.02	RI, MS, BI
Limonene	1030	1029	$C_{10}H_{16}$	138–86–3	0.41 ± 0.02	RI, MS, BI
Bergamal	1054	1056	$C_9H_{16}O$	106–72–9	0.51 ± 0.02	RI, MS
γ-Terpinene	1059	1059	$C_{10}H_{16}$	99–85–4	0.27 ± 0.01	RI, MS, BI
Linalool	1103	1096	$C_{10}H_{18}O$	78–70–6	0.74 ± 0.06	RI, MS, BI
cis-Rose oxide	1110	1108	$C_{10}H_{18}O$	4610–11–1	0.15 ± 0.01	RI, MS, BI
trans-Rose oxide	1129	1125	$C_{10}H_{18}O$	5258–11–7	0.14 ± 0.01	RI, MS, BI
Dihydrolinalool	1136	1135	$C_{10}H_{20}O$	18 479–51–1	0.47 ± 0.03	RI, MS, BI
Isopulegol	1148	1149	$C_{10}H_{18}O$	121 468–66–4	2.91 ± 0.16	RI, MS, BI
Citronellal	1153	1153	$C_{10}H_{18}O$	106–23–0	40.99 ± 2.60	RI, MS, BI
Neoiso-isopulegol	1174	1171	$C_{10}H_{18}O$	21 290–09–5	0.13 ± 0.01	RI, MS, BI
Myrtenal	1179	1195	$C_{10}H_{14}O$	564–94–3	0.06 ± 0.01	RI, MS, BI
Terpinen-4-ol	1181	1177	$C_{10}H_{18}O$	562–74–3	0.05 ± 0.00	RI, MS, BI
α-Terpineol	1194	1188	$C_{10}H_{18}O$	98–55–5	0.07 ± 0.01	RI, MS, BI
Citronellol	1227	1225	$C_{10}H_{20}O$	106–22–9	14.61 ± 0.68	RI, MS, BI
Neral	1241	1238	$C_{10}H_{16}O$	106–26–3	0.33 ± 0.01	RI, MS, BI
Geraniol	1259	1252	$C_{10}H_{18}O$	106–24–1	0.81 ± 0.04	RI, MS, BI
Methyl citronellate	1264	1261	$C_{11}H_{20}O_2$	2270–60–2	0.22 ± 0.01	RI, MS
Geranial	1274	1267	$C_{10}H_{16}O$	141–27–5	0.37 ± 0.00	RI, MS, BI
Citronellyl formate	1278	1273	$C_{11}H_{20}O_2$	105–85–1	0.06 ± 0.00	RI, MS, BI
Bornyl acetate	1288	1285	$C_{12}H_{20}O_2$	76–49–3	0.07 ± 0.01	RI, MS, BI
Menthanyl acetate	1300	1300	$C_{12}H_{20}O_2$	20 777–41–7	0.09 ± 0.01	RI, MS, BI
γ-Pyronene	1338	1345	$C_{10}H_{16}$	514–95–4	0.09 ± 0.00	RI, MS
α-Cubebene	1351	1351	$C_{15}H_{24}$	17 699–14–8	0.69 ± 0.03	RI, MS, BI
Citronellyl acetate	1358	1352	$C_{12}H_{22}O_2$	150–84–5	3.33 ± 0.16	RI, MS, BI
Cyclosativene	1368	1371	$C_{15}H_{24}$	22 469–52–9	0.13 ± 0.03	RI, MS, BI
α-Copaene	1378	1374	$C_{15}H_{24}$	3856–25–5	3.42 ± 0.16	RI, MS, BI
Geranyl acetate	1387	1381	$C_{12}H_{20}O_2$	105–87–3	0.11 ± 0.01	RI, MS, BI
β-Cubebene	1391	1388	$C_{15}H_{24}$	13 744–15–5	0.35 ± 0.02	RI, MS, BI
β-Elemene	1393	1390	$C_{15}H_{24}$	515–13–9	0.35 ± 0.01	RI, MS, BI
β-Caryophyllene	1421	1419	$C_{15}H_{24}$	87–44–5	2.73 ± 0.12	RI, MS, BI
γ-Elemeno	1438	1436	$C_{15}H_{24}$	29 873–99–2	1.34 ± 0.06	RI, MS, BI
α-Guaiene	1440	1439	$C_{15}H_{24}$	3691–12–1	0.06 ± 0.01	RI, MS, BI
α-Humulene	1454	1454	$C_{15}H_{24}$	6753–98–6	0.36 ± 0.01	RI, MS, BI
Germacrene D	1482	1481	$C_{15}H_{24}$	23 986–74–5	0.73 ± 0.03	RI, MS, BI
Cubebol	1517	1515	$C_{15}H_{26}O$	23 445–02–5	0.13 ± 0.01	RI, MS, BI
δ-Cadinene	1525	1523	$C_{15}H_{24}$	483–76–1	0.29 ± 0.01	RI, MS, BI
Elemol	1552	1549	$C_{15}H_{26}O_2$	639–99–6	0.13 ± 0.01	RI, MS, BI
Germacrene B	1558	1561	$C_{15}H_{24}$	15 423–57–1	0.46 ± 0.01	RI, MS, BI
Spathulenol	1580	1578	$C_{15}H_{24}O_2$	6750–60–3	0.84 ± 0.03	RI, MS, BI
Caryophyllene oxide	1585	1583	$C_{15}H_{24}O$	1139–30–6	0.50 ± 0.02	RI, MS, BI
Guaiol	1600	1600	$C_{15}H_{26}O$	489–86–1	0.26 ± 0.01	RI, MS, BI
Rosifoliol	1610	1600	$C_{15}H_{26}O$	63 891–61–2	0.09 ± 0.01	RI, MS, BI
Vulgarone B	1652	1651	$C_{15}H_{22}O$	64 180–68–3	0.07 ± 0.00	RI, MS, BI
Bulnesol	1671	1671	$C_{15}H_{26}O$	22 451–73–6	0.38 ± 0.02	RI, MS, BI
cis,cis-Farnesol	1698	1698	$C_{15}H_{26}O$	16 106–95–9	11.95 ± 0,55	RI, MS, BI
2,3-Dihydrofarnesol	1699	1689	$C_{15}H_{28}O$	27 745–36–4	3.67 ± 0.19	RI, MS, BI
Class composition						
Monoterpene hydrocarbons				2.84		
Oxygenated monoterpenes				66.36		
Sesquiterpene hydrocarbons				10.91		
Oxygenated sesquiterpenes				18.02		
Total identification %				98.13		

RI HP-5MS, retention index on the HP-5MS column relative to C8 - C24 *n*-alkanes.
RI lit., Adams mass spectral-retention index library (Adams, 2007).
RI, identification by comparison with RI HP-5MS with those described by Adams (2007).
MS, identification by comparison with NIST 17 MS databases.
BI, identification by comparison with Babushok' s retention index (Babushok et al., 2011).
[a] Compounds are listed in order of their elution from an HP-5MS column.

quantitation or/and detection of a large number of metabolites from one or more samples. This strategy, known as top-down or metabolite profile strategy, avoids the need for a preceding detailed hypothesis on a particular set of metabolites and, instead, analyzes the total metabolomic profile in a specific complex sample. This is of paramount importance, since phytochemical approaches need to address complex chemical matrices, and are a key tool to understanding the numerous biological activities observed for substances such as EOs. Untargeted GC−MS analysis of the GcEO allowed us to identify 52 compounds (Table 1). The GcEO contains a complex mixture mainly consisting of oxygenated monoterpenes (66.36%), along with oxygenated sesquiterpenes (18.02%) and sesquiterpene hydrocarbons (10.91%) (Fig. 1).

All identified compounds represent 98.13% of the total oil, with citronellal (40.99%), citronellol (14.61%) and farnesol (11.95%) as the major constituents. Other components, present in minor quantities, were 2,3-dihydrofarnesol (3.67%), α-copaene (3.42%), citronellyl acetate (3.33%), isopulegol (2.91%), β-caryophyllene (2.73%). To the best of our knowledge, the metabolic profile of GcEO is new to the literature and the major constituents identified in this study are not commonly found in other species of *Guatteria*.

On the other hand, spathulenol, germacrene D, germacrene B, caryophyllene oxide and β-pinene have been reported as being predominant in essential oils of the leaves of *Guatteria* (Costa et al., 2020; Siqueira et al., 2015; Palazzo et al., 2008). However, these metabolites, including β-pinene, germacrene D and B, spathulenol and caryophyllene oxide, are present in the GcEO, but in minor quantities. The metabolomic content in a plant's essential oil reflects the genetic background of the species, and is affected by biotic and abiotic components of environment (Silva et al., 2021).

3.2. Antibacterial properties of the GcEO

In this study, the investigation of the GcEO demonstrated its capacity to inhibit Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Klebsiella pneumoniae* and *Salmonella enterica*) bacteria. The GcEO inhibited the growth of each pathogen assayed with minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values ranging from 5.0 to 20.0 μL mL^{-1} (Table 2). The lowest MIC value was against *B. subtilis* and *S. aureus* (both 5.0 μL mL^{-1}), followed by *K. pneumoniae* and *Sa. enterica* (both 10.0 μL mL^{-1}). Estimated IC_{50} values of the dose-response curve showed robust antibacterial activities of GcEO against *S. aureus* (IC_{50} = 2.94 μL mL^{-1}) and *B. subtilis* (IC_{50} = 3.2 μL mL^{-1}), while the IC_{50} values were 5.74 and 7.15 μL mL^{-1} for *Sa. enterica* and *K. pneumoniae*, respectively (Fig. 2A and Table 2). However, the positive control reference (norfloxacin) was more efficient than the GcEO in all assayed bacteria. Antibacterial properties have been reported previously in other *Guatteria* EOs. The EO of *G. punctata* was shown to be active against *Streptococcus mutans* and *Streptococcus pyogenes* with an MIC of 4.68 μg mL^{-1} (Bay et al., 2019). The EO from the leaves of *G. australis* exhibited a slight effect against *Staphylococcus aureus* and *Escherichia coli* (MIC 250 μg mL^{-1}). Alcântara et al. (2017) demonstrated that the *G. blepharophylla* EO exhibited activity against *Streptococcus sanguinis*, *Staphylococcus aureus* and *Enterococcus faecalis*, with MIC values of 0.02, 0.05 and 0.05 mg mL^{-1}, respectively. Regarding the GcEO, the antibacterial effect can be attributed to the dominant oxygenated monoterpenes, citronellal (40.99%) and citronellol (14.61%), which have been reported to cause disruption in the permeability of cell membranes (Abril- Sánchez et al., 2019; Guimarães et al., 2019; Singh et al., 2016) In this study, Gram-negative bacteria were less sensitive to GcEO than Gram-positive bacteria. The presence of the lipopolysaccharide outer membrane of Gram-negative bacteria can hinder the interaction of antibacterial substances, and the cytoplasmic membrane offers more resistance to the pathogenic cell (Zhang et al., 2021).

3.3. Antifungal properties of the GcEO

The inhibitory effect of the GcEO was evaluated against the plant pathogenic fungi *A. alternata*, *As. flavus, F. oxysporum* and *C. guaranicola*. The GcEO exhibited antifungal activity against four phytopathogens, with MIC and MFC values ranging from 1.25 to 10.0 μL mL^{-1}. The highest inhibitory activity was against *A. alternaria* with MIC and MFC values of 1.25 μL mL^{-1}, followed by *As. flavus* and *F. oxysporum* (both 5.0 μL mL^{-1}), and *C. guaranicola* (10.0 μL mL^{-1}). IC_{50} values estimated by using a nonlinear function (Fig. 2B and Table 3) revealed the highest inhibitory effect against *A. alternaria,* with a value of 0.67 μL mL^{-1}, while the IC_{50} value of *C. guaranicola* was 5.1 μL mL^{-1}, which is a 7.6-fold difference between these IC_{50}.

There is no previous description of the effect of the GcEO on the vegetative growth of phytopathogenic fungi. Furthermore, *Guatteria* EOs remain unexplored in terms of their ability to inhibit these pathogens. However, Annonaceae EOs have already been explored from this point of view. Tegang et al. (2018) found promising antifungal activity against *As. niger* and *F. oxysporium* using *Xylopia aethiopica* EO and reported β-pinene as the main compound. *Duguetia lanceolata* EO showed a fungicidal effect against *As. flavus* in a dose-dependent manner (Ribeiro et al., 2020).

In general, EOs are exceptional sources of biomolecules against phytopathogenic fungi, and have great potential in control strategies for fungal damage. A number of studies have reported the antifungal activity of EOs, which include encapsulated EOs and purified molecules from EOs (Razola-Díaz et al., 2021). Recently, Al-Ansari et al. (2021) reported that EO extracted from *Lavandula latifolia* exhibited marked antifungal activity against *Trichophyton mentagrophytes*, *F. oxysporum*, *Rhizoctonia solani* and *As. nidulans*. The antifungal mechanism of EOs seems to involve the disturbance of cell membrane integrity, which leads to irreversible damage of the membrane and leakage of cell contents (Perumal et al., 2021; Souza et al., 2020). Since, among the fungi tested, *A. Alternaria* was the most sensitive to the GcEO, it was selected to evaluate the mode of action of the GcEO.

3.4. Mode of action of the GcEO on A. Alternaria conidia

Conidia are vital vehicles for reproduction, dispersal and survival of

Table 2
Antimicrobial activity of the GcEO against pathogenic bacteria.

	GcEO					Norfloxacin*				
Strain	**MIC**	**MBC**	**IC_{50} (95% CI)**	**R^2**	**RMSE**	**MIC**	**MBC**	**IC_{50} (95% CI)**	**R^2**	**RMSE**
B. subtilis	5.0	5.0	3.55 (3.20–4.01)	0.97	1.83	0.62	0.62	0.07 (0.06–0.08)	0.97	6.05
S. aureus	5.0	10.0	2.94 (2.44–3.52)	0.95	7.79	0.62	0.62	0.11(0.09–0.13)	0.91	6.23
K. pneumoniae	10.0	20.0	7.15 (6.04–8.48)	0.95	6.83	0.62	0.62	0.10 (0.09–0.11)	0.99	1.85
S. enterica	10.0	20.0	5.74 (5.16–6.37)	0.98	4.78	0.62	0.62	0.06 (0.05–0.05)	0.93	3.82

MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) were expressed in μL mL- 1. *Norfloxacin (1 mg mL- 1) was used as the positive control.

IC_{50}, concentration (μL mL^{-1}) that causes 50% inhibition of fungal growth. 95% CI, 95% confidence intervals, the values are considered significantly different when the 95% CI fails to overlap. R^2, coefficient of determination. RMSE, root mean square error.

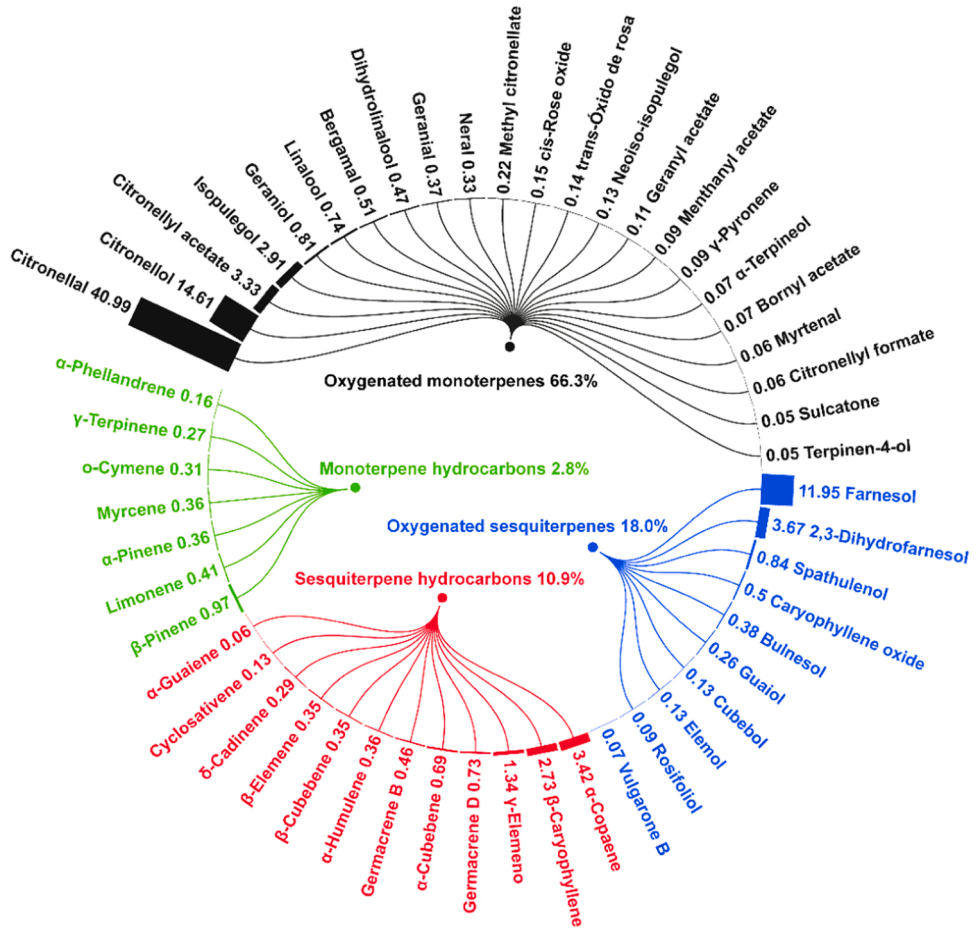

Fig. 1. Radial treemap of the chemical constituents of GcEO analyzed using GC–MS. The compound names are followed by the relative peak areas (%).

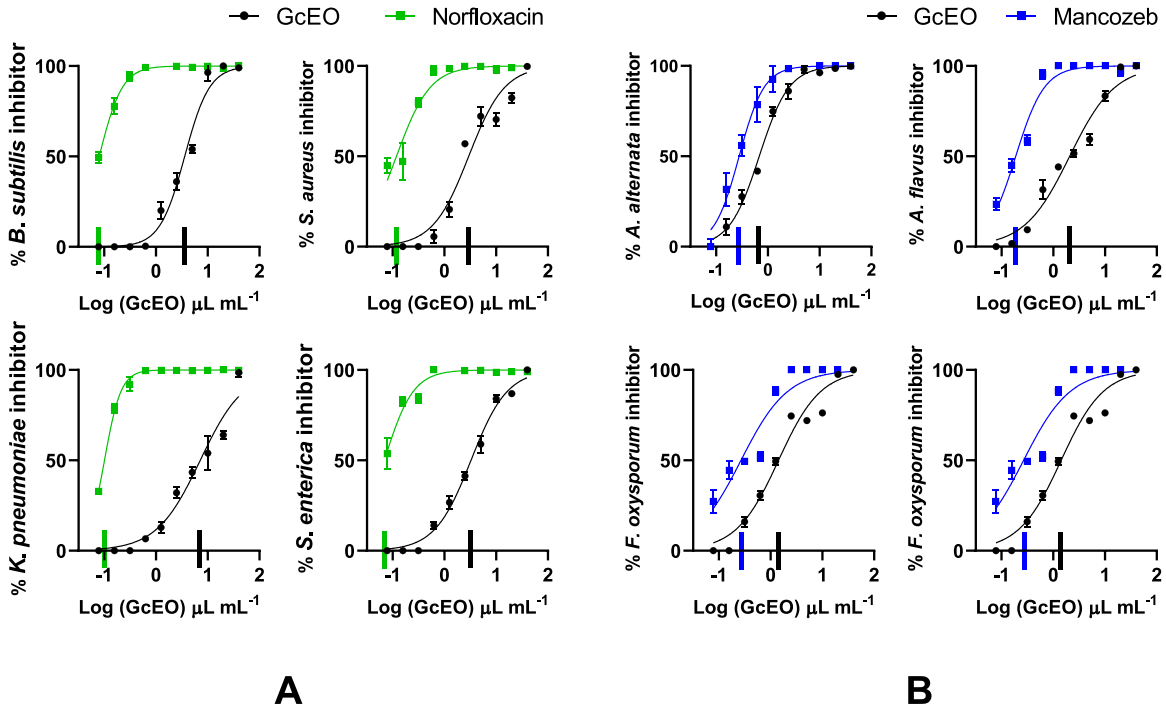

Fig. 2. Representative dose-response curves of the antimicrobial activity of GcEO against pathogenic bacteria (A) and phytopathogenic fungi (B). Extra-long ticks (black for GcEO; green for norfloxacim and blue for mancozeb) on the x-axis represent the $LogIC_{50}$ valor. Data are presented as the mean ± standard deviation ($n = 3$).

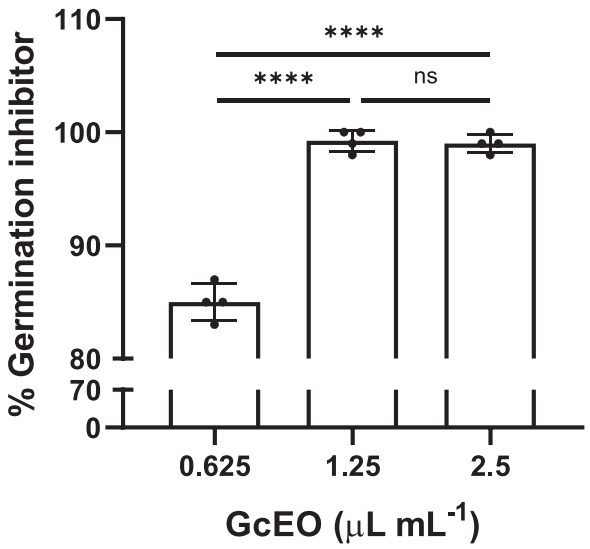

Fig. 3. Inhibition of conidial germination of *Alternaria alternata* by GcEO. Data are presented as the mean ± standard deviation ($n = 4$) of three experiments. ns = nonsignificant. * ** * $p < 0.0001$ indicates statistical difference using ANOVA and Tukey's post hoc test.

fungi, and can cause substantial economic losses. There is a great demand from the agro-food industry for the discovery of bioagents capable of preventing, mitigating or controlling the contamination of food and plants (Matrose et al., 2021; Polozsányi et al., 2021). In this study, the mechanism of action of the GcEO was evaluated based on the integrity of cell membranes and structural alterations in *A. alternata* conidia.

First, the capacity of different concentrations (0.625, 1.25 and 2.5 μL mL^{-1}) matching to 1/2 ×MIC, MIC and 2 ×MIC values of the GcEO to inhibit the germination of conidia was investigated. The germination of *A. alternata* conidia treated with the GcEO for 24 h was noticeably inhibited (> 99%) at 1.25 and 2.5 μL mL^{-1}, while at 0.625 μL mL^{-1} the reduction of germination was around 85% (Fig. 3). The treatments with 1.25 and 2.5 μL mL^{-1} were more effective than the treatment with 0.625 μL mL^{-1} ($p > 0.0001$). The use of EOs as an antifungal agent has been widely reported in the literature, and studies report that these substances are effective in inhibiting germination (Black-Solis et al., 2019; Peralta-Ruiz et al., 2020). Inhibition of conidia germination is a promising strategy to prevent fungal infection and, thus, reduce disease severity (Gorai et al., 2021).

Many reports show that the content of nucleic acid and protein in a fungal suspension is a significant indicator of the loss of integrity of cell membranes (Li et al., 2021). The results showed that GcEO induced significant leakage of nucleic acids from *A. alternata* conidia (Fig. 4A). The absorbance values for nucleic acids (A_{260} nm) of GcEO-treated conidia were superior to those of the control group ($p < 0.001$). The A_{260} nm values at 0.625, 1.25 and 2.5 μL mL^{-1} concentrations ranged from 0.54 ± 0.02–0.64 ± 0.01, while the negative control was 0.15 ± 0.02. At 1.25 and 2.5 μL mL^{-1} concentrations, A_{260} nm values were higher than 0.625 μL mL^{-1}, thus indicating a notably dose-dependent leakage.

Similarly, in evaluating the release of soluble proteins, there was a difference between the GcEO-treated conidia and the control group ($p < 0.001$) (Fig. 4B). The soluble protein values in suspension of the treatments with 1.25 μL mL^{-1} (191.2 ± 20.34 μgP mL^{-1}) and 2.5 μL mL^{-1} of GcEO (206.6 ± 11.84 μgP mL^{-1}) were approximately 9.5 and 10.3 times that of the control group (20.1 ± 4.37 μgP mL^{-1}), respectively. At the 0.625 μL mL^{-1} concentration of GcEO, the increase in protein release was lower when compared to the 1.25 μL mL^{-1} ($p < 0.05$) and 2.5 μL mL^{-1} ($p < 0.001$). The effects of the GcEO on the release of soluble proteins were consistent with those on the release of nucleic acids. These results indicated that the integrity of the *A. alternata* conidia membrane was indeed affected by the GcEO. The proposed antifungal mechanisms for EOs implicate in the capacity of their components to pass through the cell wall and cause damage to the integrity of the cytoplasmic membrane, which results in the leakage of cell material, cellular collapse and cell death (Hu et al., 2021; Kong et al., 2021).

In an additional strategy for studying the antifungal mechanism of the GcEO, the morphological alterations of *A. alternata* conidia were evaluated using microscopy. Under light microscopy, untreated conidia presented a normal germination with the development of hyphae, while GcEO-treated *A. alternata* conidia were completely inhibited (Fig. 5A and B). However, alterations in the morphology of the conidia were not perceptible when using this technique. SEM analysis of the morphology of *A. alternata* showed differences between GcEO-treated and non-

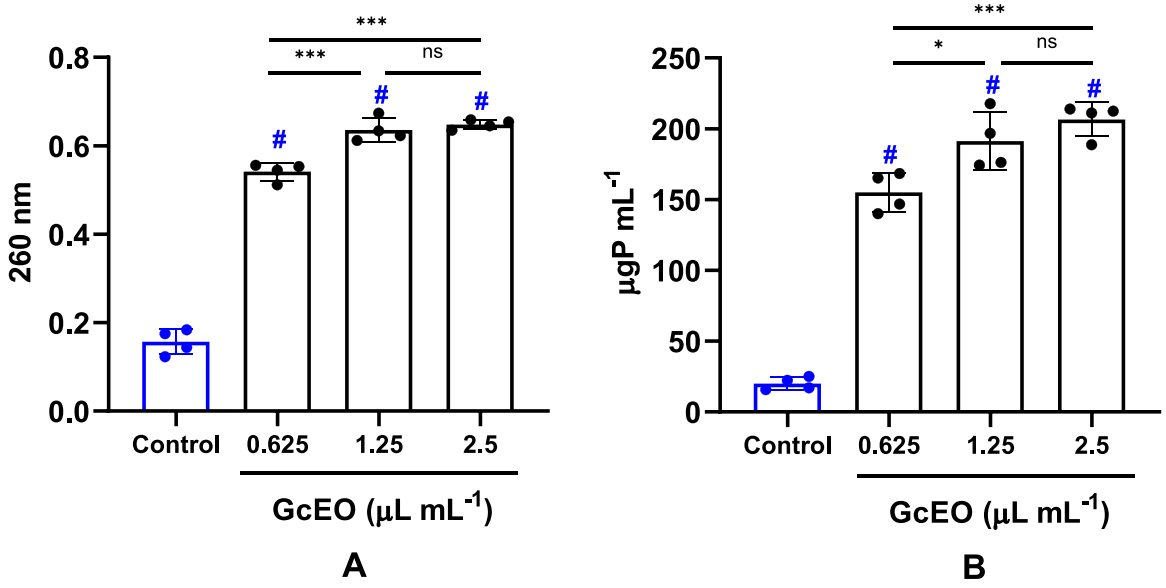

Fig. 4. Effects of GcEO on leakages of nucleic acids (A) and soluble protein (B) in *Alternaria alternata* conidia. Data are presented as the mean ± standard deviation ($n = 4$) of three experiments. ns = nonsignificant. # $p < 0.0001$ compared with control (0.1% Tween-80), * $p < 0.05$ and * ** $p < 0.001$ indicate statistical difference by ANOVA and Tukey's post hoc test.

Table 3
Antimicrobial activity of the GcEO against phytopathogenic fungi.

	GcEO					Mancozeb*				
Strain	**MIC**	**MFC**	**IC_{50} (95% CI)**	**R^2**	**RMSE**	**MIC**	**MFC**	**IC_{50} (95% CI)**	**R^2**	**RMSE**
A. alternata	1.25	1.25	0.67 (0.63–0.73)	0.99	3.69	1.25	1.25	0.27 (0.24–0.3)	0.97	5.17
As. flavus	5.0	5.0	2.06 (1.74–2.47)	0.96	6.77	1.25	1.25	0.18 (0.16–0.2)	0.96	5.09
F. oxysporum	5.0	5.0	1.41 (1.2–1.67)	0.96	6.82	1.25	1.25	0.26 (0.21–0.33)	0.91	8.1
C. guaranicola	10.0	10.0	5.1 (4.71–5.51)	0.95	5.57	1.25	1.25	0.19 (0.16–0.22)	0.95	5.78

MIC (minimal inhibitory concentration) and MFC (minimal fungicidal concentration) expressed in μL mL- 1. *Mancozeb (2 mg mL- 1) was used as the positive control. IC_{50}, concentration (μL mL^{-1}) that causes 50% inhibition of fungal growth. 95% CI, 95% confidence interval, the values are considered significantly different when the 95% CI fails to overlap. R^2, coefficient of determination. RMSE, root mean square error.

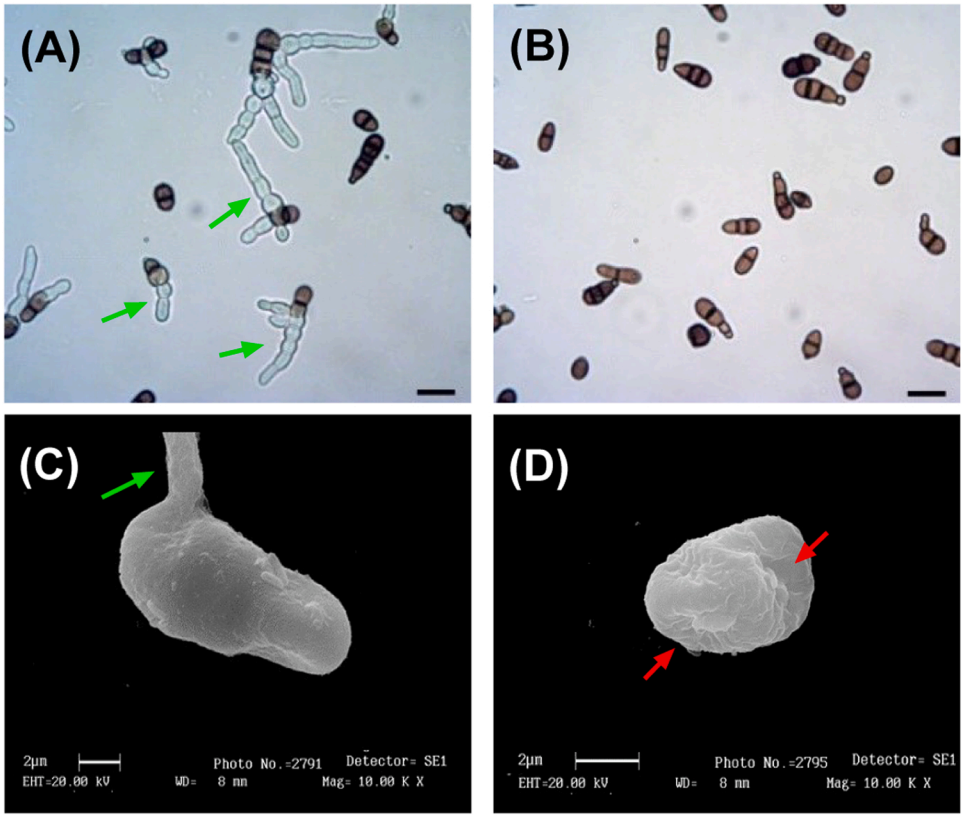

Fig. 5. Micrographs of *Alternaria alternata* conidia after treatment with GcEO. (A) Light micrographs of non-treated conidia. (B) Light micrographs of GcEO-treated conidia at MIC level. (C) Scanning electron micrograph of non-treated conidia. (D) Scanning electron micrograph of GcEO-treated conidia at 1.25 μL mL^{-1} concentration (MIC level). Green arrows indicate the germination of conidia. Red arrows indicate conidia shrinkage and wrinkling. A and B, bar represents 20 μm. C and D, bar represents 2 μm.

treated conidia (Fig. 5C and D). The treatment with 1.25 μL mL^{-1} caused severe damage, and the cell surface became deformed, wrinkled and sunken. Some authors have suggested that the deformed and wrinkled surface of conidia occurs through the rupture of membrane integrity and loss of the cytoplasmic contents and the blockage of cell growth (Behbahani et al., 2019; Guo et al., 2020). In this context, specific cell markers (proteins and nucleic acids) were released at multi-fold levels that were higher than in the control. Furthermore, evident structural changes were observed in GcEO-treated conidia.

Some researchers have attributed the antifungal effect to the key compounds present in EOs, usually the most abundant compounds (Pimentel et al., 2018; Rguez et al., 2018; Wang et al., 2018). In the GcEO, citronellal and citronellol are the most abundant substances, and have already been described as having strong antifungal properties (Aguiar et al., 2014; Barbosa et al., 2016; Kaur et al., 2021; Lee et al., 2008; Tolba et al., 2015). Citronellal is a monoterpenoid found in more than 50 aromatic plants; however, it is mainly extracted from the leaves of *Corymbia citriodora* (Myrtaceae). It is an unsaturated aldehyde with a chiral center, which renders it a chemically reactive molecule (Araújo-Filho et al., 2018; Goodine and Oelgemöller, 2020). Studies with citronellal-rich EOs have shown that these EOs have deleterious effects against multiple fungi (Dhakad et al., 2018). Morcia et al. (2017), reported potent antifungal activity of citronellal against *F. sporotrichioides*, *F. graminearum* and *F. langsethiae*. A recent study by Ouyang et al. (2021) concluded that the citronellal effectively reduced *Penicillium digitatum* infection in citrus fruits. Furthermore, the exposure of *P. digitatum* to citronellal led to convincing cell membrane damage. Wu et al. (2016) reported that citronellal can lead to increased release of cellular constituents due to plasma membrane damage.

Citronellol, the second most abundant substance found in GcEO, is an acyclic chiral primary alcohol that contains a double bond, and which confers stability to the molecule. This monoterpene occurs naturally in various aromatic plant species (Santos et al., 2019). Antifungal activity of citronellol has been reported against *Trichophyton rubrum*, and appears to involve damage and loss of integrity of the cytoplasm membrane (Pereira et al., 2015). This compound exhibits a strong antifungal

effect against *Botryosphaeria dothidea* (Zhang et al., 2018). Citronellol can inhibit both mycelial growth and conidial germination of *C. fructicola* and *C. acutatum* in a dose-dependent manner, and its mechanism can be related to disturbance of membrane fluidity and permeability (Scariot et al., 2020). These reports afford evidence that corroborates with data obtained in our study, i.e., that essential oils and their constituents act on membrane integrity and permeability of fungi.

In addition to citronellal and citronellol, the GcEO is composed of multiple molecules in minor concentrations (including farnesol, citronellyl acetate, β-caryophyllene, caryophyllene oxide and α-pinene). In general, the antimicrobial effect of EOs containing these molecules is well documented (Allenspach and Steuer, 2021; Liu et al., 2018; Lopes et al., 2021; Santos et al., 2021), and it is reasonable to hypothesize that this myriad of active molecules results in a synergistic action among its components, which enhances the protective effect of the EO. Therefore, the EO can act in different plant defense mechanisms against several stress situations (Langat et al., 2021; Zengin and Baysal, 2014). As such, further in-depth studies are needed to examine the complex mechanisms and the synergistic effects of the compounds, and their use as in vivo bioactive agents.

4. Conclusions

This study shows that the EO isolated from the leaves of *G. citriodora* has a complex and differentiated metabolomic profile and demonstrated the presence of the most important classes of compounds, highlighting the oxygenated monoterpenes citronellal and citronellol. In addition, in dose-dependent manner, this study demonstrated antibacterial and antifungal activity of the EO against all the pathogens analyzed. The results obtained herein revealed that GcEO treatment was able to suppress the germination of *A. alternata* conidia, induce the release of specific cell markers (e.g., nucleic acids and protein) and alter the morphology of conidia, which was associated with the damage to cellular membranes system. The GcEO appears to be membrane-active on *A. alternata* conidia, which results in the modification of the membrane's properties and function. Therefore, GcEO is potential candidate as a biodegradable, ecofriendly antifungal agent, and stands out due to the biologic potential of the results presented here.

Funding

This study was financed in part by the Coordination for the Improvement of Higher-Level Personnel - Brazil (CAPES) - Finance Code 001, Amazonas State Research Support Foundation (FAPEAM, Brazil, 024/2014), the São Paulo Research Foundation (FAPESP, Brazil, grant 2020/03390-8) and by the National Council for Scientific and Technological Development (CNPq, Brazil, 308373/2019-7) for financial support of this study. José F. C. Gonçalves acknowledges the fellowship provided by CNPq.

CRediT authorship contribution statement

D**P Souza and JFC Gonçalves**: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Funding acquisition, Supervision. **JC de Carvalho, KKG da Silva and AV Fernandes:** Methodology, Formal analysis. **GO Nascimento, MV Ramos, HHF Koolen, DP Bezerra and AS Santos:** Methodology, Data analysis, Writing – original draft. **DP Souza, JFC Gonçalves, JC de Carvalho, KKG da Silva, AV Fernandes, MV Ramos, HHF Koolen, DP Bezerra and AS Santos**: Final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Ministry of Science, Technology and Innovations/National Institute for Amazonian Research (MCTI/INPA - Brazil), as well as the members of the Laboratory of Physiology and Plant Biochemistry of INPA (INPA-LFBV) for the use of the equipment and infrastructure for the analyses and to the Coordination for the Improvement of Higher Education Personnel (CAPES, project Pro-Amazonia: Biodiversity and Sustainability - 042/2012) and National Council for Scientific and Technological Development (CNPq) for financial support. J.F.C Gonçalves, M. V. Ramos, H. H. F. Koolen, D.P. Bezerra and A.S. Santos thank (CNPq-Brazil) for PQ research fellowships.

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