

Article

Antifungal and Allelopathic Effects of Essential Oil from *Calyptanthes concinna* DC. Dried Leaves and of Its Major Constituent Elemicin

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Abstract: Essential oils (EOs) are natural products widely used in sustainable agrochemistry, not only because they are biodegradable and safe but also because they are regarded as alternatives to chemical fungicides against fungal species that attack crops. Allelopathy, another field of study, falls within the most recent and sustainable strategies applied to weed suppression to replace synthetic herbicides. Therefore, this study reports the chemical composition and allelopathic and antifungal effects of the EOs extracted from *Calyptanthes concinna* dried leaves (Cc-EO) and its pure major constituent elemicin. Their antifungal activities were evaluated by the disk diffusion method (DDM) at doses between 0.05 mg/mL and 0.4 mg/mL of Cc-EO and elemicin. The allelopathic effect was evaluated by studying the inhibition of germination and the growth of *Lactuca sativa* seeds. The chemical composition of Cc-EO was determined by GC-MS and GC-FID analyses. The major constituents of Cc-EO were elemicin (60.5%), α -cadinol (9.0%) and caryophyllene oxide (8.3%). Cc-EO and elemicin were assayed in vitro against 17 fungi of agronomic interest (*Aspergillus niger*, *A. flavus*, *A. nomius*, *Penicillium digitatum*, *P. expansum*, *Sclerotinia sclerotiorum*, *S. rolfsii*, *S. minor*, *Fusarium graminearum*, *Myrothecium verrucaria*, *Corynespora cassicola*, *Erwinia psidii*, *Colletotrichum musae*, *Alternaria carthami*, *Rhizoctonia solani*, *Rhizopus stolonifer* and *Macrophomina phaseolina*). The concentration of Cc-EO (0.4 mg/mL) inhibited 100% of the mycelium growth of seven strains, equal to the fungicide fluazinam, which was used as a positive control. Elemicin showed antifungal activity against all fungi at all concentrations under investigation (above 50%). A strong allelopathic effect was recorded for Cc-EO and elemicin at the dose of 0.28 mg/mL, with the almost total inhibition of germination. This study revealed, for the first time, the strong and remarkable fungicidal and allelopathic effects of Cc-EO and elemicin, an important finding for the agrochemical field.

Keywords: *guamirim*; *guamirim-facho*; mycelium growth; bioherbicide; elemicin; natural active ingredients



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1. Introduction

The increased demand for food to feed the ever-growing population has led to the development and adoption of synthetic chemicals as a quick and effective strategy for managing crop pests and diseases [1]. However, overreliance on synthetic pesticides is discouraged due to their detrimental effects on human health, the environment, and the development of resistant pest and pathogen strains [1]. As a result of the increasingly incorrect and indiscriminate use of toxic agrochemical products for decades, several problems, such as the accumulation of toxic residues in food, water and soil contamination, the intoxication of farmers, the selection of resistant pests, and the ending of the system of biological control using natural enemies leading to the outbreak of pests and diseases that

affect human beings, have emerged [2]. The negative effects caused by toxic agrochemicals have concerned scientists all over the world and have made them search for safe and sustainable alternatives tirelessly [3].

The field of agrochemistry, which uses knowledge regarding the chemistry of natural products, has led to a significant increase in studies on compounds that are not aggressive to human health and the environment. These studies have been triggered by a great demand for healthy food products that do not have pesticide residues [4]. In this scenario, plants are fundamental since some produce essential oils (EOs) that are not only highly active against an array of biological targets, but are also used as crude extracts. EOs and extracts of plants are promising sources of bioactive molecules and natural inputs that can be by the industry to formulate safe products of agronomic interest [5].

EOs, also known as volatile oils (VOs) extracted from aromatic plants, have been defined as a complex mixture composed of 20–60 constituents that have a low molecular mass and evaporate easily [6]. Their constituents may belong to different classes of secondary metabolites, such as monoterpenes, sesquiterpenes and phenylpropanoids [6]. Since EOs are biologically active, they act against different biological targets; as a result, their remarkable antibacterial, antifungal and allelopathic activities have already been reported [6]. The literature reports that their satisfactory biological activities are closely related to their major constituents; however, this does not exclude the synergic effect that also exists in all identified constituents [6].

The aromatic plant called *Calypttranthes concinna* belongs to the family Myrtaceae; it was chosen to be the target of this study because there are few reports about its EOs in the literature. In addition, plants that belong to this family, including Brazilian species, have been acclaimed in the literature because they produce highly active EOs [7]. The genus *concinna* has 100 species and some of their plants have molecules with the chromene group, which is responsible for several activities, such as antibacterial, anti-inflammatory and antiparasitical ones [8]. *C. concinna*, which is a tree with a grayish tortuous trunk that may reach 5 m, is called *guamirim-facho*, *guamirim* and *camboim-ferro* in Brazilian Portuguese [9].

This is the first comprehensive study of the chemical composition of EOs extracted from *C. concinna* dried leaves and their antifungal and allelopathic activities of agronomic interest. EOs have been known to and applied in agrochemistry because they act against phytopathogenic fungi that harm important crops, such as corn, soybeans and beans, and cause great economic losses [10]. Evaluating the allelopathic activities of EOs is relevant to agriculture since they may act on the seeds of weeds, affecting growth, harming development and inhibiting germination. Allelopathic activities enable us to determine whether EOs may act as herbicides, a fact that would contribute significantly to sustainable agriculture and the control of weeds [11].

As part of our ongoing project on the biological activities of EOs [12] and mainly because this study is the first one showing the agrochemical application of Cc-EOs *in vitro*, this study aimed to investigate the antifungal activities of Cc-EOs and pure elemicin against 17 fungal strains of agronomic interest and to determine their allelopathic effect by studying the inhibition of germination and the growth of *L. sativa* seeds. In addition, it aimed to identify volatile constituents of Cc-EO by gas chromatography–mass spectrometry (GC-MS) and gas chromatography–flame ionization detection (GC-FID).

2. Material and Methods

2.1. Plant Material and EO Extraction

C. concinna DC. adult leaves of a single plant (Figure 1) were collected in Iporá, Goiás (GO) state, Brazil (16°28'00" S and 51°09'00" W), in April 2016 in the rainy autumn season. They were identified by the biologist Erika Amaral, and a voucher specimen of *C. concinna* (CC2301-Iporá) was deposited at the Herbarium Jataiense by Professor Germano Guarim Neto. Leaves were then taken to the Laboratory of Natural Product Chemistry at IF Goiano-Campus Rio Verde, located in Rio Verde, GO. The leaves were weighed and dehydrated in an air circulation oven at 40 °C for 24 h.



Figure 1. *C. concinna* DC. leaves (Myrtaceae).

The samples of *C. concinna* dried leaves (3×300 g) were added to a 1 L round-bottom flask containing 500 mL of distilled water and subjected to hydrodistillation in a Clevenger-type apparatus for 3 h. After the manual collection of the EO samples, traces of remaining water in the EOs were removed with anhydrous sodium sulfate, which was followed by filtration. The isolated oil (Cc-EO) was stored under refrigeration until the analysis and assays. The calculation of the Cc-EO yield was based on dried leaves (w/w).

Pure elemicin (1 g) was purchased from Biosynth® in October 2016 (CAS no. 487-11-6; Product Cod FE22655).

2.2. GC-MS and GC-FID Analyses

Gas chromatography–flame ionization detection (GC-FID) analyses were performed by a Shimadzu GC2010 plus gas chromatograph equipped with an AOC-20 s autosampler and fitted with a flame ionization detector (FID) and a data-handling processor. An Rtx-5 (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30-m $\times$ 0.25-mm i.d.; 0.25- μ m film thickness) was employed. The operating conditions were the following: the column temperature was programmed to rise from 60 to 240 °C at 3 °C/min and then held at 240 °C for 5 min; carrier gas = He (99.999%), at 1.0 mL/min; injection mode; injection volume, 0.1 μ L (split ratio of 1:10); and injector and detector temperatures = 240 and 280 °C, respectively. Relative concentrations of the EO components were obtained by peak area normalization (%) and expressed as the mean of three replicate analyses.

GC-MS analyses were carried out by a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column was an RTX-5MS (Restek Co., Bellefonte, PA, USA) with a fused silica capillary (30 m $\times$ 0.25 mm i.d., $\times$ 0.25 μ m film thickness). The electron ionization mode occurred at 70 eV. Helium (99.999%) was employed as the carrier gas at a constant flow of 1.0 mL/min. The injection volume was 0.1 μ L (split ratio of 1:10). The injector and ion-source temperatures were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC. Mass spectra were taken at a scan interval of 0.5 s, in the mass range of 40 to 600 Da. The identification of the Cc-EO constituents was based on their retention indices on an RTX-5MS capillary column under the same operating conditions as those used in the GC-FID analyses, related to a homologous series of n-alkanes (C_8 – C_{40}). The structures were computer-matched with the Wiley 7, NIST 08 and FFNSC 1.2 spectra libraries and their fragmentation patterns were compared with data in the literature [13].

2.3. Antifungal Assays

The following 17 strains were used in this study: *Aspergillus niger*, *A. flavus*, *A. nomius*, *Penicillium digitatum*, *P. expansum*, *Sclerotinia sclerotiorum*, *S. rolfsii*, *S. minor*, *Fusarium graminearum*, *Myrothecium verrucaria*, *Corynespora cassiicola*, *Erwinia psidii*, *Colletotrichum musae*, *Alternaria carthami*, *Rhizoctonia solani*, *Rhizopus stolonifer* and *Macrophomina phaseolina*. They were supplied by the Empresa Brasileira de Pesquisa Agropecuária “Embrapa Arroz e Feijão” and taken to the laboratory for the isolation of microorganisms using the method devised by Sahi and Khalid [14]. Fungi were collected in infected plants, including soybeans (*Aspergillus flavus*, *Aspergillus nomius*, *Sclerotinia sclerotiorum*, *S. rolfsii*, *S. minor*, *Corynespora cassiicola*, *Alternaria carthami*, *Rhizoctonia solani* and *Macrophomina phaseolina*); orange (*Penicillium digitatum*); apple (*P. expansum*); onion (*Aspergillus niger*); wheat (*Fusarium graminearum*); cotton (*Myrothecium verrucaria*); guava (*Erwinia psidii*); papaya (*Colletotrichum musae*); and strawberry (*Rhizopus stolonifer*). Parts of the infected plants were cut into small pieces (1.5 cm in length) and all surfaces were sterilized with H₂O₂ at 15% for 45 s. Afterwards, all surfaces were rinsed three times with distilled water. Then, they were placed onto 2% Potato Dextrose Agar (PDA) medium in Petri plates and incubated at 25 °C. After six days, fungal isolates appearing on the parts of the plants were identified and transferred to 2% PDA medium Petri plates for purification. To prevent bacterial contamination, PDA was supplemented with fluazinam (0.19 g/L) and the pH was adjusted to 6.5. All plates were incubated at 25 ± 2 °C for 3–7 days. Developed colonies were sub-cultured on fresh PDA by hyphal tip and/or the single spore isolation technique proposed by Kobori et al. [15]. Re-isolation from artificially diseased plants was performed. Developed colonies were sub-cultured on fresh PDA and identification was confirmed with the original isolates to achieve Koch’s postulations. The isolates of all fungi were maintained on PDA slants and refrigerated at 5 °C as stock cultures for further studies. An in vitro assay was performed to evaluate the activities of Cc-EO and elemicin against the mycelium growth of pathogenic fungi by the direct method described by Adjou et al. [16]. Various amounts of standard Cc-EO and elemicin were added individually to conical flasks containing 100 mL of PDA prior to solidification and then gently shaken to give the required concentrations (0.05, 0.1, 0.2, 0.3 and 0.4 mg/mL). A fungicide (fluazinam, 0.19 g/L) was added to the medium to avoid fungicidal contamination. The poisoned medium was poured on sterile Petri dishes at the rate of 25 mL per plate and allowed to solidify. Plates were individually inoculated at the center with equal disks (7 mm) taken from 7-day-old fungal cultures. Plates inoculated with fungi without any botanicals were retained as the control. Each treatment was carried out three times. All plates were incubated at 28 °C until the assayed fungi reached full growth in the control. The mycelium growth size was measured by averaging the two dimensions of each fungal colony. The Inhibition of Mycelium Growth (IMG) was calculated using the formula proposed by Pinto et al. [17]:

$$\text{IMG \%} = [(dc - dt/dc) \times 100]$$

dc = mycelium growth size in control and dt = mycelium growth size in treatment.

2.4. Statistical Analysis

Data on IMG were subject to analysis of variance (ANOVA). Means were compared by the Fisher’s test with protected least significant difference (LSD) at $p < 0.05$. The package heatmaply was used for preparing the heat map figure in RStudio Version 2023.03.0 + 386.

2.5. Allelopathic Assays

The allelopathic activities of Cc-EO and elemicin were evaluated against lettuce seeds in agreement with Jalaei et al., with slight modifications [18]. Commercial lettuce (*Lactuca sativa*) seeds were surface sterilized with 70% ethanol for 30 s, washed with sterile water to remove ethanol and then disinfected with 0.2% sodium hypochlorite solution for 20 min, followed by three rinses of distilled water for 5 min each. After disinfection, 30 seeds

were placed on glass Petri dishes covered with Whatman no. 1 filter paper. Seeds were soaked with 10 mL of each Cc-EO and the elemicin concentrations prepared in dimethyl sulfoxide (DMSO) (1%, *v/v*). The Petri dishes were sealed with parafilm and incubated in a climate room at 25 ± 1 °C with a photoperiod of 12:12 for 10 days [18]. Experiments were performed in quadruplet and the control containing DMSO 1% was added. Observations and measurements of the germination rate of seeds and the growth of lettuce seedlings were carried out. The plant growth in the incubation period was expressed as shoot length (cm) at the end of the period. The shoots per plate were measured and the allelopathic inhibition of the shoot growth was calculated by comparing it with the control, as follows:

Inhibition rate (%) = $[100\% \times (\text{length of shoots in control} - \text{length of shoots in treatment}) / \text{length of shoots in control}]$ [19,20].

3. Results and Discussion

3.1. Chemical Composition of Cc-EO

The EO from *C. concinna* dried leaves (Cc-EO) was light-yellowish and the yield was $0.2 \pm 0.01\%$. Twenty compounds were identified in Cc-EO, mainly elemicin (60.5%, 1), α -cadinol (9.0%, 2) and caryophyllene oxide (8.3%, 3), at high concentrations (Figure 2). Other constituents that were also found at considerable concentrations ($\geq 6.0\%$) were spathulenol and τ -cadinol (Table 1). The chemical composition found in this study is similar to the one found by Antonelo et al. (2022) [21]. They also identified that the concentration of the major constituent—elemicin—was 36.43%. This difference—almost 24%—may be due to two reasons. Firstly, *C. concinna* leaves were collected in different Brazilian states, i.e., Antonelo et al. (2022) [21] collected them in Paraná while this study collected them in GO. Secondly, in this study, leaves were collected, dried and then subjected to the process of EO extraction, while the researchers from Paraná extracted them from fresh leaves. The Cc-EO from fresh leaves was also studied in the state of Rio Grande do Sul; its chemical composition exhibited β -bisabolene (25.5%) as the major constituent but the phenylpropanoid elemicin was not identified [22]. The findings of the study conducted by Costa et al. (2020) [8] agree with the ones obtained in the study carried out by Antonelo et al. (2021) [21] and those reported in this paper. Elemicin (17.9%) was also the major constituent in the study conducted by Costa et al. (2020) [8]. It should be highlighted that the Cc-EO extracted from species found in GO is a promising source of elemicin at high concentrations. The chemical differences exhibited by the EOs result from several factors, such as biotic, abiotic and genetic ones [23]. Differences in the relative amounts of volatile constituents in Cc-EOs are explained by the process used to dry the leaves before they are subject to oil extraction. The literature states that the drying process may directly affect the composition and yield of EOs, but the results depend on every species and the drying method [24].

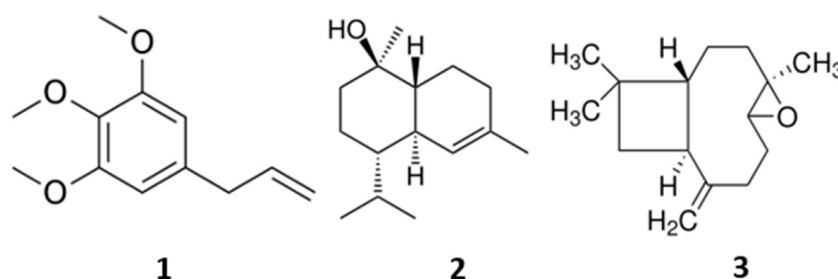


Figure 2. Major volatile constituents of Cc-EO: elemicin (60.5%, 1), α -cadinol (9.0%, 2) and caryophyllene oxide (8.3%, 3).

Table 1. Chemical composition of EOs from *C. concinna* (Cc-EO) determined by GC-FID and GC-MS.

Compounds	Retention Index (RI)		
	RI _{exp}	RI _{lit}	RA%
β-Pinene	971	973	0.2
Sabinene	980	980	1.0
cis-β-Ocimene	1005	1007	0.1
Copaene	1338	1339	0.3
β-bourbonene	1382	1382	0.7
E-Caryophyllene	1416	1417	0.5
Aromadendrene	1441	1441	1.5
α-Humulene	1448	1450	0.8
γ-Muurolene	1477	1479	1.0
α-Amorphene	1483	1483	1.0
β-Selinene	1489	1489	0.1
Bicyclogermacrene	1496	1497	0.5
δ-Amorphene	1509	1511	1.0
Elemicin	1555	1555	60.5
Spathulenol	1577	1577	6.2
Caryophyllene oxide	1580	1581	8.3
Globulol	1589	1590	1.2
Viridiflorol	1590	1590	0.1
τ-Cadinol	1638	1640	6.0
α-Cadinol	1653	1653	9.0
Total			100

RI_{lit}: Retention Index [13]; RA%: relative area. Bold numbers mean that those volatile constituents were considered major constituents of Cc-EO.

3.2. Antifungal Effects of Cc-EO and Elemicin against Phytopathogenic Fungi

In their evolutionary process, plants develop several active compounds with biological activities, such as EOs, which have antimicrobial potential that may inhibit fungal growth [25]. This is the first report of the in vitro antifungal activities of Cc-EO and its major constituent, elemicin, in the literature. Its results revealed the remarkable antifungal activities of Cc-EO (Table 2) since both Cc-EO and elemicin (Table 3) exhibited a high potential to inhibit the growth of the seventeen strains under investigation. The fungicide fluazinam was the positive control because its commercial trademark Frowncide 500SC had already been used by other studies to investigate the antifungal activities of natural products [26,27].

Table 2. Inhibition of Mycelium Growth (IMG) of EO from *C. concinna* leaves (Cc-EO) against seventeen fungi of agronomic interest.

Fungal Strains	IMG Concentrations (mg/mL) of Cc-EO (%)				
	0.05	0.1	0.2	0.3	0.4
<i>Aspergillus niger</i>	45.2 ± 6.0	65.0 ± 2.8	70.3 ± 1.0	82.3 ± 0.1	100 ± 0.0
<i>Aspergillus flavus</i>	46.5 ± 7.6	67.0 ± 2.0	77.9 ± 0.2	87.4 ± 1.0	100 ± 0.0
<i>Aspergillus nomius</i>	26.3 ± 24.8	53.1 ± 7.4	65.8 ± 3.0	78.3 ± 0.2	91.4 ± 0.8
<i>Penicillium digitatum</i>	43.8 ± 4.3	53.8 ± 7.0	70.0 ± 1.0	85.5 ± 1.2	100 ± 0.0
<i>Penicillium expansum</i>	13.5 ± 13.0	25.9 ± 3.0	37.9 ± 0.6	50.2 ± 8.0	68.3 ± 0.7
<i>Sclerotinia sclerotiorum</i>	13.2 ± 2.7	27.8 ± 2.0	41.7 ± 3.0	52.4 ± 3.0	71.3 ± 2.0
<i>Sclerotinia rolfsii</i>	3.7 ± 0.8	12.4 ± 0.2	25.6 ± 0.7	37.1 ± 1.0	54.6 ± 1.0
<i>Sclerotinia minor</i>	18.6 ± 8.4	28.5 ± 3.5	36.5 ± 0.3	48.4 ± 0.3	61.7 ± 0.1

Table 2. Cont.

Fungal Strains	IMG Concentrations (mg/mL) of Cc-EO (%)				
	0.05	0.1	0.2	0.3	0.4
<i>Fusarium graminearum</i>	7.1 ± 5.1	20.3 ± 2.0	31.1 ± 0.1	45.3 ± 0.1	60.0 ± 3.0
<i>Myrothecium verrucaria</i>	10.3 ± 2.6	20.0 ± 1.0	32.4 ± 0.8	46.0 ± 0.1	67.8 ± 0.5
<i>Corynespora cassiicola</i>	49.9 ± 15.5	55.9 ± 0.4	69.8 ± 0.1	80.0 ± 0.1	100 ± 0.0
<i>Erwinia psidii</i>	14.0 ± 2.6	29.3 ± 5.0	39.9 ± 3.0	50.0 ± 5.0	54.2 ± 7.0
<i>Colletotrichum musae</i>	50.0 ± 5.0	60.3 ± 3.0	68.3 ± 2.0	80.3 ± 3.0	100 ± 0.0
<i>Alternaria carthami</i>	43.8 ± 4.3	57.4 ± 2.0	66.7 ± 1.0	80.0 ± 0.1	100 ± 0.0
<i>Rhizoctonia solani</i>	20.9 ± 4.2	38.8 ± 3.0	49.7 ± 0.2	60.0 ± 0.3	68.3 ± 1.0
<i>Rhizopus stolonifer</i>	17.3 ± 3.0	27.3 ± 0.2	40.0 ± 1.3	67.9 ± 3.0	87.5 ± 2.0
<i>Macrophomina phaseolina</i>	46.3 ± 6.0	58.0 ± 1.3	69.0 ± 0.7	81.9 ± 0.3	100 ± 0.0
Fluazinam *	100 ± 0.0				

Data are expressed as means ± standard error ($n = 3$). * Positive control. Bold numbers highlight total fungal inhibition.

Table 3. Inhibition of Mycelium Growth (IMG) of elemicin against seventeen fungi of agronomic interest.

Fungal Strains	IMG Concentrations (mg/mL) of Elemicin (%)				
	0.05	0.1	0.2	0.3	0.4
<i>Aspergillus niger</i>	51.0 ± 2.0	60.0 ± 0.1	79.5 ± 2.0	81.0 ± 0.1	89.3 ± 0.1
<i>Aspergillus flavus</i>	51.2 ± 1.0	62.5 ± 3.0	78.1 ± 1.0	83.2 ± 3.0	90.5 ± 0.5
<i>Aspergillus nomius</i>	53.0 ± 1.2	61.0 ± 0.1	75.4 ± 0.4	85.5 ± 0.3	93.0 ± 0.2
<i>Penicillium digitatum</i>	53.5 ± 4.0	60.5 ± 1.0	69.3 ± 2.0	71.3 ± 2.0	80.3 ± 1.0
<i>Penicillium expansum</i>	51.1 ± 0.3	67.5 ± 0.3	77.3 ± 1.0	87.4 ± 0.1	95.5 ± 0.1
<i>Sclerotinia sclerotiorum</i>	50.8 ± 4.0	57.8 ± 1.0	70.1 ± 0.2	78.9 ± 3.0	87.2 ± 3.0
<i>Sclerotinia rolfsii</i>	57.0 ± 1.0	65.9 ± 2.0	72.3 ± 0.6	82.0 ± 2.0	91.8 ± 0.3
<i>Sclerotinia minor</i>	55.0 ± 0.3	63.4 ± 1.0	75.9 ± 1.0	87.1 ± 0.3	96.1 ± 0.1
<i>Fusarium graminearum</i>	52.0 ± 3.0	63.0 ± 0.2	70.6 ± 2.0	77.3 ± 1.0	83.7 ± 3.0
<i>Myrothecium verrucaria</i>	56.3 ± 2.0	65.9 ± 2.0	73.4 ± 0.5	79.0 ± 5.0	84.5 ± 0.1
<i>Corynespora cassiicola</i>	51.0 ± 0.1	60.1 ± 1.2	67.9 ± 2.0	76.9 ± 2.0	82.3 ± 3.0
<i>Erwinia psidii</i>	52.3 ± 2.0	62.6 ± 2.0	73.1 ± 2.0	83.2 ± 0.5	92.1 ± 0.4
<i>Colletotrichum musae</i>	58.0 ± 4.0	67.0 ± 1.0	78.0 ± 3.0	85.6 ± 0.1	95.3 ± 1.0
<i>Alternaria carthami</i>	55.2 ± 1.0	64.4 ± 2.0	72.2 ± 0.1	80.9 ± 2.0	90.1 ± 0.2
<i>Rhizoctonia solani</i>	54.0 ± 1.0	68.3 ± 2.0	76.6 ± 2.0	87.9 ± 0.8	98.0 ± 1.4
<i>Rhizopus stolonifer</i>	56.0 ± 0.4	65.0 ± 3.0	73.9 ± 1.0	83.3 ± 2.0	92.3 ± 0.1
<i>Macrophomina phaseolina</i>	57.7 ± 0.1	69.9 ± 2.0	77.8 ± 2.0	88.4 ± 1.0	99.3 ± 0.3
Fluazinam *	100 ± 0.0				

Data are expressed as means ± standard error ($n = 3$). * Positive control. Bold numbers highlight fungus inhibition above 90%.

Cc-EO at 0.4 mg/mL completely inhibited mycelium growth in the following fungus species: *Aspergillus niger*, *A. flavus*, *Penicillium digitatum*, *Corynespora cassiicola*, *Colletotrichum musae*, *Alternaria carthami* and *Macrophomina phaseolina*. At the same dose, Cc-EO also satisfactorily inhibited the growth of *Aspergillus nomius* (91.4%), followed by *Rhizopus stolonifer*, whose inhibition was 87.5%. On the other hand, other strains such as *S. rolfsii* and

E. psidii were found to be more resistant to the activities of Cc-EO; both exhibited values of IMG slightly above 50% at the highest dose under study.

The antifungal activities of EOs may be attributed to the fact that they inhibit pectinesterase (PME) and affect the degree of methyl esterification in pectines, the main components of fungal cell walls [28]. Changes in the integrity of fungal cell walls enables EOs, which are easily permeable, to penetrate cell membranes, promote cell lysis and, consequently, kill microorganisms [28,29].

Studies of Cc-EOs have added new findings to research that has established the potential use of medicinal and aromatic plants as biofungicides in the agricultural sector [30]. Cc-EO had its antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* described by Costa et al. [8]. Its antioxidant activity, which was determined by DPPH, ABTS and FRAP, was found interesting [21]. Finally, Brazilian plants that belong to the family Myrtaceae have often been subject to phytochemical and pharmacological studies due to their secondary metabolites and bioactive EOs [31].

Inhibition assays carried out with the pure constituent elemicin (Figure 2) showed its satisfactory antifungal activity, i.e., approximately 50% of the mycelium growth of all strains was inhibited, even at the lowest concentration of 0.1 mg/mL (Table 3). All inhibition values above 90% are in bold in Table 3 to highlight the fungi that are most sensitive to elemicin at 0.4 mg/mL: *Aspergillus flavus*, *A. nomius*, *Penicillium expansum*, *Sclerotinia rolfii*, *Sclerotinia minor*, *Erwinia psidii*, *Colletotrichum musae*, *Alternaria carthami*, *Rhizoctonia solani*, *Rhizopus stolonifer* and *Macrophomina phaseolina*. Tables 2 and 3 show that Cc-EO and elemicin acted as expected against all fungi under evaluation, since there was an increase in the activity of IMG when the concentrations of samples increased; hence, a dose–response relationship with antifungal activity is shown.

Previous studies have shown that elemicin is responsible for antifungal activity against *Colletotrichum acutatum*, the agent that causes anthracnose [32]. It is also active against the fungus that attacks coffee beans (*Cladosporium cladosporioides*) [33]. Elemicin is one of the major constituents of EOs from *Boesenbergia pulcherrima* roots, acting as a powerful oil against *Fusarium oxysporum* [34]. It has also exhibited good antifungal activity against *Candida tropicalis* and *Aspergillus flavus* [35]. EOs from *Daucus carota* ssp. *halophilus* were shown to possess significant antifungal activity against dermatophyte strains due to elemicin [36]. Thus, plant extracts and EOs rich in elemicin and its biosynthetic derivatives may be considered agroactive natural sources [37].

3.3. Allelopathic Effects of Cc-EO and Elemicin against *L. sativa* Seeds

The allelopathic effects of Cc-EO and elemicin were evaluated according to the germination percentage and shoot growth (cm) on *L. sativa* seeds. The results showed (Tables 4 and 5) that Cc-EO and elemicin had a significant allelopathic effect on the germination and shoot growth of the seeds under study (*L. sativa*) in a dose-dependent manner, in comparison with the control.

Table 4. Inhibitory effect of Cc-EO on seed germination and shoot height of lettuce.

Cc-EO Concentrations (mg/mL)	Shoot Growth (cm)	Germination (%)
Control	2.35 ± 0.07	100
0.018	2.33 ± 0.11	45.8 ± 0.1
0.035	1.84 ± 0.12	16.7 ± 0.5
0.07	1.05 ± 0.15	7.9 ± 0.05
0.14	0.55 ± 0.1	4.3 ± 0.02
0.28	0.30 ± 0.02	2.5 ± 0.1

Data are shown as means ($n = 30$) ± SE (standard errors). The bold number highlights a high level of germination inhibition (97.5%).

Table 5. Inhibitory effect of elemicin on seed germination and shoot height of lettuce.

Elemicin Concentrations (mg/mL)	Shoot Growth (cm)	Germination (%)
Control	2.35 ± 0.07	100
0.018	2.20 ± 0.15	45.0 ± 0.1
0.035	1.70 ± 0.01	15.5 ± 0.5
0.07	0.98 ± 0.03	6.2 ± 0.05
0.14	0.40 ± 0.1	3.3 ± 0.1
0.28	0.25 ± 0.02	2.1 ± 0.1

Data are shown as means ($n = 30$) ± SE (standard errors). The bold number highlights a high level of germination inhibition (97.9%).

The allelopathic effects exhibited by Cc-EO and elemicin were similar and equally promising at 0.28 mg/mL. It may be stated that the allelopathic effect of Cc-EO results from the high concentration of elemicin in the crude oil. The results of this study seem to be the first data on the allelopathic activities of Cc-EO and elemicin published in the literature. The allelopathic properties of plants and their allelochemicals play a fundamental role in sustainability, a fact that has been supported by several studies of agroecology [38]. Therefore, based on in vitro assays, this study suggests that Cc-EO and elemicin should be considered for use in herbicides and as strong candidates for further in vivo assays.

Finally, the mechanisms implicated in the allelochemical activity of different EOs have been often attributed to high concentrations of monoterpenes that affect plant germination and growth since they lead to morphological and physiological changes in plants, such as the inhibition of the respiration chain of the mitochondrial matrix, the inhibition of mitosis, changes in the integrity of cell membranes, the deterioration of cuticular waxes, an increase in transpiration, lipid peroxidation and damage to microtubules [39]. One study found that the monoterpenes present in EOs hinder photosynthesis and do not enable germination and growth to take place because they decrease respiratory activity [40]. A remark should also be made about the other two major constituents of Cc-EO, i.e., α -cadinol and caryophyllene oxide. Both terpene constituents also contribute to the phytotoxic activity of Cc-EO since they exhibit known allelopathic activities [40,41].

4. Conclusions

In the constant search for environmentally friendly compounds with antifungal and herbicide effects, this study contributed to revealing, for the first time, the promising effects of EOs from *C. concinna* dried leaves and their major constituent elemicin. Cc-EO proved to be a rich source of elemicin. The EO extracted from *C. concinna* dried leaves exhibited a higher content of elemicin than the one extracted from its fresh leaves. It is worth mentioning that the other major constituents found in Cc-EO were α -cadinol and caryophyllene oxide. Cc-EO was highly active against the following phytopathogenic fungi: *Aspergillus niger*, *A. flavus*, *Penicillium digitatum*, *Corynespora cassiicola*, *Colletotrichum musae*, *Alternaria carthami* and *Macrophomina phaseolina*; this is because 100% of the mycelium growth was inhibited. Pure elemicin was able to inhibit approximately 90% of the mycelium growth of ten different fungi. On the other hand, Cc-EO and elemicin exhibited similar and satisfactory allelopathic effects at all the concentrations under evaluation. The results of this study have added to knowledge about the role of the monoterpene composition as a natural product that provides new and important biological agents at the forefront of integrated weed management strategies. In order to confirm the usefulness of these results, the next step will be the testing of Cc-EO and the interaction of elemicin with soil under greenhouse and field conditions.

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