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Article

Antimicrobial Activity of the Essential Oil from *Croton pluriglandulosus* Carn. Leaves against Microorganisms of Clinical Interest

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Abstract: Multiresistant pathogens pose a serious threat to human health. The genus *candida* is one class of human pathogenic yeasts responsible for infections affecting healthy and immunocompromised patients. In this context, plant essential oils emerged as a future natural alternative to control the diseases caused by these pathogens. Here, it highlighted antimicrobial activity and the mechanisms of action of the essential oil extracted from *Croton pluriglandulosus* Carn.-Torres & Riina (*CpEO*) leaves on human pathogenic microorganisms in planktonic and biofilm lifestyles. In addition, for the first time, the oil composition was revealed by GC-MS analysis and the toxicity to human red blood cells (HRBC). Twenty-six chemical compounds were identified in *EOCp*, Elemicin, Bicyclogermacrene, caryophyllene, brevifolin and 2,4,6-trimethoxy-styrene. Through hemolytic assay, it was shown that *CpEO* has no toxicity to human RBCs. At the concentration of 50 μ g mL⁻¹, *CpEO* did not show great antibacterial potential. However, promising data were found for *C. krusei* and *C. parapsilosis* inhibiting by 89.3% and 80.7% of planktonic cell growth and 83.5% and 77.9% the biofilm formation, respectively. Furthermore, the mechanisms of action *CpEO* were elucidated by fluorescence. Scanning electron microscopy revealed damage to the cell membrane and pore formation, ROS overproduction, and Induction of apoptosis in *candida* cells. Our results reinforce the potential of *CpEO* as an effective alternative molecule of pharmaceutical interest.

Keywords: croton; yeast; inhibition; chemical composition and action mechanisms

1. Introduction

Pathogens seriously threaten human health and generate global concern due to drug resistance. The genus *Candida* holds many species, including *Candida albicans*, the most common and prevalent human pathogenic yeast [1]. However, recent studies emphasize increasing clinical cases by non-*albicans* yeasts, such as *C. glabrata*, *C. krusei*, and *C. lusitaniae*, affecting healthy and

immunocompromised patients [2,3]. The infection establishment can be non-invasive or invasive, causing local and systemic manifestations [4].

The treatment of such infections is made with antifungal agents that directly interfere with the cell structure and metabolism of the fungus. However, due to the misuse, these fungi acquired resistance and became less susceptible to conventional drugs [5]. One form of resistance is the formation of biofilms, which are aggregates of microbial cells embedded in an extracellular polymer matrix that adheres to surfaces and exhibits drug resistance [6].

Plants are a source of promising alternative molecules to control infections. Among these molecules are the secondary metabolites with antimicrobial action [7,8]. One of the natural products with antifungal action is essential oils (EOs). Hydrophobic and volatile substances are usually obtained through plants' plant organs (Leaves, Stem, Root and fruits) [9].

The genus *Croton*, from the Euphorbiaceae family, adapted to the Caatinga biome, is recognized for grouping diverse species with the potential for producing essential oils and active ingredients. These plants are widely used in folk medicine as teas to reduce pain, intestinal problems, and as anti-inflammatory [10].

In addition, some species are aromatic and produce essential oils with antifungal, insecticidal and antimicrobial properties [11]. Among them *C. zehntneri*; *C. pulegioides* Baill; *C. jacobinensis* Baill; *C. nepetaefolius* and *C. blanchetianus* show activity against human pathogens [3,10,12,13].

Despite this large amount of data on *Croton* species, works with essential oils and their biological applications are still scarce. An example is the *C. pluriglandulosus* Carn.-Torres & Riina, for which the only information reported in the literature is about the characteristics of the plant, such as being classified as a shrub and the morphology of its leaves as ovate. It has many trichomes on the leaves, latex, and acropeculiar glands. In Brazil, it is predominantly found in regions with the caatinga biome [14].

Given the phytotherapeutic and microbial potential of species belonging to the *Croton* genus, the present work sought to understand and determine the activity and mechanisms of action of the essential oil extracted from the leaves of *Croton pluriglandulosus* (CpOE) Carn.-Torres & Riina, which is poorly studied regarding the control of microorganisms in planktonic and biofilm form.

2. Methodology

2.1. Biological material

C. albicans (ATCC 10231), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC22019), and the bacteria *B. subtilis* (ATCC 6633), *Enterobacter aerogenes* (ATCC 13048), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus* (ATCC 25923), and *Salmonella enterica* (ATCC 14028) were all obtained from the Laboratory of Plant Toxins, Federal University of Ceará, Brazil.

2.2. Plant material and extraction of essential oil

The harvest of the leaves of *C. pluriglandulosus* Carn. (CpEO) were performed in Serra do Lima, in the city of Patu in Rio Grande do Norte (6° 06' 36" S, 37° 38' 12" O) state. A voucher specimen was deposited in the Dáardano de Andrade Lima Herbarium of the Federal University of Rural Semiarid (UFERSA), with volume number MOSS 15058. The technique used for oil extraction was based on Oliveira et al. [15], with modifications established by Malveira et al. [16]. Fresh leaves of CpEO (approximately 630 g) were subjected to hydrodistillation in a clevenger apparatus for 2 h. The fresh leaf biomass was weighed and placed in a 5 L volumetric flask containing 2 L of distilled water. The extracted oil was dehydrated with ether and anhydrous sodium sulfate and stored in an amber glass bottle under refrigeration until analysis.

2.3. Characterization of CpEO by GC/MS Analysis

Identification of the major chemical compounds of CpEO was performed by gas chromatography coupled to a mass spectrometer (GC/MS) analysis (Shimadzu GCMS-QP2010 SE, Kyoto, Japan),

equipped with an Rtx®-5MS capillary column (30 m × 0.25 mm × 0.25 µm. The *Cp*EO (55 µL) was placed in a vial (2 mL vial with 250 µL inserts) and injected for data acquisition. The operating conditions of the GC-MS/MS were optimized as follows: 70 eV, carrier gas (He), a flow rate of 1.7 mL·min⁻¹ and a pressure of 53.5 KPa. Injector and detector interface temperatures were 25 °C and 230 °C, respectively. The oven temperature program was 100 °C for 3 min⁻¹ and then 310 °C at a heating rate of 3.5 °C min⁻¹ and maintained at 310 °C for 5 min⁻¹. The identification of the constituents of the essential oils was investigated by comparing the mass spectra and Kovats index (IK) values with those of the research reference library.

2.4. Antimicrobial activity

The anticandida assay with adaptations was performed using the microdilution test based on the methodology described by the Clinical and Laboratory Standards Institute (CLST, 2008). Colonies were suspended in Sabouraud Dextrose medium (twice concentrated) and diluted to (2.5 × 10⁶ CFU mL⁻¹). For antimicrobial evaluation, aliquots of 50 µL of medium and yeast cells were added to wells of 96-well plates along with 50 µL of *Cp*EO (ranging from 50 to 0.008 µg mL⁻¹). Itraconazole antifungal and DMSO 5% negative control was used as the positive control. The plates were incubated for 24 h at 30 °C. The yeast development was monitored using a microplate reader (Epoch, Bio-Tek Instruments) at an absorbance of 600 nm. The antibacterial susceptibility assay was based on the method determined by Oliveira et al. [17], with modifications. The Mueller-Hinton Broth medium was used to grow the strains. The experimental setup followed the same principles as the test mentioned above, with only the positive control using ciprofloxacin.

2.5. Antibiofilm assay

For the evaluation of the inhibition of biofilm formation, the assay was based method described by Dias et al. [18]. The experimental strategy, *Cp*EO concentration and assay controls with the planktonic cells. At 24 h, the supernatant was removed and washed three times with 0.15 M NaCl. Next, 200 µL of 100% methyl alcohol for 15 min at 37 °C was added to fix the cells. A 0.1% crystal violet solution (200 µL) was added and incubated for 30 min. Then, the wells were washed with distilled water and 200 µL of 33% acetic acid was added for 15 min. Finally, it was read at an absorbance of 590 nm using a plate reader.

2.6. Mechanisms of Action

2.6.1. Cell membrane integrity

The minimum inhibitory concentration was selected to be evaluated for mechanisms of action. According to Oliveira et al. [19], cell membrane integrity was analyzed with modifications. For the experiment, propidium iodide (PI) was implemented to evaluate *Cp*EO-induced pore formation. First, 50 µL of *C. krusei* and *C. parapsilosis* (2.5 × 10⁶ CFU mL⁻¹, in Saboraud liquid medium) were added in the presence of 50 µL of *Cp*EO (50 µg mL⁻¹) and incubated for 24 h at a temperature of 30 °C. Subsequently, each treatment was washed with 100 µL of 0.15 M NaCl and centrifuged (5000 xg, 5 min at 4 °C) three times. The samples were resuspended in 50 µL of 0.15 M NaCl and 3 µL of 1 mM propidium iodide (PI) and incubated for 30 min in the dark at 37 °C. Finally, all treated were washed twice with 0.15 M NaCl, centrifuged to remove excess PI, and observed under a fluorescence microscope (Olympus System BX 60) with an excitation wavelength of 585 nm and emission wavelength 617 nm.

2.6.2. Detection of ROS Overproduction

To evaluate *Cp*EO-induced ROS generation, the method described by Dikalov and Harrison [20], with modifications, was used. All samples were prepared as previously described, with samples incorporated 9 µL of 0.2 mM DCFH-DA (2',7'-Dichlorofluorescein diacetate) were observed under a

microscope (Olympus System BX60) using an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.6.3. Induction of apoptosis

The Induction of apoptosis in the species studied was based on using CellEvent (ThermoFisher), following the manufacturer's restrictions for detecting caspase action. Samples were prepared following the same pattern described in the previous tests and 9 μ L of CellEvent® reagent (ThermoFisher, São Paulo, SP, Brazil) was added. Finally, washes and centrifugations were performed as before. Finally, the treatments were observed with a microscope (Olympus System BX60) using an excitation wavelength of 342 nm and an emission wavelength of 441 nm.

2.7. Scanning Electron Microscopy (SEM)

SEM (Billerica, MA, USA) analysis was performed to evaluate cell morphological changes based on the method described by Staniszewska et al. [21]. The cells were treated under the same conditions described in the previous sections, then fixed with 1% (v/v) glutaraldehyde in 0.15 M sodium phosphate buffer at pH 7.2 for 16 h at room temperature. This was followed by three washes with sodium phosphate buffer pH 7.2 and centrifuged (5000g for 5 min at 4 °C). To dehydrate, the samples were dried with ethanol (30%, 50%, 70%, 100%, 100% (v/v) for 10 min each and centrifuged as above. Finally, 50% hexamethyldisilane (HMDS, Sigma, St. Louis, MI, USA) was diluted in ethanol, left for 10 min, followed by centrifugation, and 1000% HMDS was added. 15 μ L was transferred to a coverslip to dry at room temperature. The cells were coated with a gold layer with an aluminum surface using a positron emission tomography (PET) coating machine (Emitech-Q150TES, Quorum Technologies, Lewes, England) and the images observed on a scanning electron microscope (Quanta 450 FEG, FEI, Waltham, MA).

2.8. Hemolytic analysis

Hemolytic analysis of *CpEO* was evaluated with human erythrocyte blood types A, B, and O, as Oliveira et al. [19] described. Three concentrations were tested, including the one used in all the assays mentioned above. Blood was centrifuged at 5000 g for 5 min at 10 °C, washed with 0.15 M NaCl and diluted to a concentration of 2.5%. Each blood type was incubated (300 μ L) with the *CpEO* solution (300 μ L) at concentrations of 1, 3, and 5 mg mL⁻¹ for 30 min at 37 °C. DMSO-NaCl (5%) and 0.1% Triton X-100 (100 %) were used as negative and positive controls for hemolysis. The samples were centrifuged (5000 g for 5 min at 4 °C, Eppendorf 5810 centrifuge, Hannover, Germany). Then the supernatants were collected and directed into 96-well plates, and the hemolysis (%) was calculated by an absorbance of 414 nm using a microplate reader.

2.9. Statistical Analysis

Three independent replicates were performed for all analyses performed. Data were subjected to the application of the ANOVA test, followed by Tukey's method using GraphPad Prism software version 5.01 (GraphPad Software company, Santa Clara, CA, USA). A $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. GC-MS/MS analysis *CpEO* by

The GC-MS/MS analysis identified 26 metabolites in *CpEO*. Elemicin (25.77%), Bicyclogermacrene (9.37%), caryophyllene (8.99%), brevifolin (4.60%) and 2,4,6-trimethoxy-styrene (3.96%) represent the compounds in the highest amount (Table 1). Many metabolites identified in *CpEO* belong to the terpenoid family, classified as monoterpenes and sesquiterpenes and present several biological activities, including antimicrobial activity [22]. Elemicin has only a few reports in the literature about its potential against human pathogens; however, Rossi et al. [23] revealed the

antimicrobial activity against the bacterium *Campylobacter jejuni*. Bicyclogermacrene and 2,4,6-trimethoxy-styrene, both compounds found in *CpEO*, have antifungal, antioxidant, larvicidal and anti-inflammatory activities [24–26].

Table 1. Main chemical components of *C. pluriglandulosus* essential oil by GC-MS analysis.

	RT	Compound	Area (%)
1	5.994	p-cymene	0.69
2	6.133	Eucalyptol (1,8-cineole)	3.75
3	9.101	epoxy-ocimene	0.21
4	10.134	4-terpineol	0.31
5	10.528	α -terpineol	2.65
6	14.611	δ -elemene	2.86
7	15.918	β -elemene	0.17
8	16.508	methyl eugenol	3.02
9	16.741	α -cis-bergamotene	0.15
10	16.894	caryophyllene	8.99
11	17.251	α -trans-bergamotene	0.87
12	17.675	neryl acetone	0.09
13	17.763	α -humulene	1.44
14	17.953	allo-aromadendrene	0.44
15	18.926	Bicyclogermacrene	9.37
16	20.193	hedycaryol	0.21
17	20.547	Elemicin	25.77
18	20.977	spathulenol	2.96
19	21.121	epiglobulol	2.17
20	21.329	globulol	0.31
21	21.937	2,4,6-trimethoxy-styrene	3.96
22	22.782	epi- α -muurolol	0.46
23	23.228	brevifolin	4.60
24	23.460	α -bisabolol	0.17
25	24.232	2Z,6Z-farnesol	0.54
26	24.705	2Z,6E-farnesal	0.28
Total identified			73.44

RT - Retention time (min); Area (%) - Relative area percentage.

Some compounds exhibit anticandidal activity. One example is eucalyptol and α -terpineol (Table 1). According to the literature, eucalyptol exhibits anti-inflammatory, antioxidant, antimicrobial and anticandidal action [27,28]. Studies show that α -terpineol has activity against yeasts and filamentous fungi, including *Candida* species (PINTO et al., 2014). Besides, β -elemene, 4-terpineol and δ -elemene were associated with antifungal, antibacterial, and anticancer activities [29,30]. Many of the components identified in *CpEO* are present in other species of the genus *Croton*, however, in different quantities [16]. This difference is related to factors such as location, climate, stage of plant development and the organ used for the study [31–33].

3.2. Antimicrobial Activity of *CpEO*

The inhibitory activity of *CpEO* (50 μ g mL⁻¹) against fungal and bacterial strains is shown in Table 2. The *CpEO* was not effective against bacterial pathogens (Table 1). In a recent study, Malveira et al. [16] reported that the essential oil from *C. blanchetianus* is ineffective against bacteria.

Nevertheless, a significant reduction in the growth of two candida species tested was observed, 89.3% for *C. krusei* and 80.7% for *C. parapsilosis* at a concentration of 50 $\mu\text{g mL}^{-1}$. The inhibition of biofilm formation for the same microorganisms was 83.5 for *C. krusei* and 77.9% for *C. parapsilosis*, respectively. The search for alternatives to treating infectious diseases caused by yeasts from the *Candida* genus has a significant impact, mainly because of the antifungal resistance, which is rapidly developing due to the indiscriminate use of antifungal agents [34]. In this context, plant essential oils emerged as a promising alternative for treating these pathogens.

Table 2. Antimicrobial effects of *C. pluriglandulosus* Carn. essential oil against planktonic cells and biofilm.

Microorganisms	<i>C_bEO (50 $\mu\text{g mL}^{-1}$)</i>			
	<i>Anticandidal Activity</i>			
	Inhibition of Planktonic cells growth (%)	Inhibition of biofilm formation (%)	Biomass reduction of preformed biofilm (%)	Itraconazole
<i>C. albicans</i>	00.00	00.00	00.00	00.00
<i>C. krusei</i>	89.3 \pm 0.02	83.5 \pm 0.01	00.00	74.5 \pm 0.05
<i>C. parapsilosis</i>	80.70 \pm 0.03	77.9 \pm 0.07	00.00	80.0 \pm 0.03
<i>Antibacterial Activity</i>				Ciprofloxacia
<i>B. subtilis</i>	00.00	00.00	00.00	67.5 \pm 0.06
<i>E. aerogenes</i>	00.00	00.00	00.00	78.7 \pm 0.02
<i>E. coli</i>	00.00	00.00	00.00	68.5 \pm 0.007
<i>K. pneumoniae</i>	8.76 \pm 0.09	00.00	00.00	72.8 \pm 0.06
<i>P. aeruginosa</i>	14.92 \pm 0.04	00.00	00.00	77.3 \pm 0.07
<i>S. aureus</i>	14.30 \pm 0.01	00.00	00.00	87.2 \pm 0.07
<i>S. enterica</i>	6.07 \pm 0.01	00.00	00.00	62.3 \pm 0.01

Malveira et al. [16] reported that the essential oil of *C. blanchetianus* possesses activity against *C. albicans* and *C. parapsilosis* in planktonic cells and biofilm using the same concentration tested in this study. Asdade et al. [35] showed the antifungal action of *Vitex agnus-castus* L. essential oil, rich in monoterpenes and sesquiterpene compounds, against clinical *Candida* strains. Taweechaisupapong et al. [36] also obtained satisfactory results using lemongrass (*Cymbopogon flexuosus*) oil aiming at biofilm inhibition of *Candida* isolates at 0.8 and 0.4 mg mL^{-1} , concentrations 16 and 8 times higher the concentration presented by CpEO.

A study with *C. rhamnifolioides* oil revealed activity against *Aeromonas hydrophila*, *E. coli*, *Listeria monocytogenes*, *S. Enteritidis*, and *S. aureus* [37]. Filho et al. [38] reported that the oil of the *Himatanthus obovatus* flowers was efficient against *C. krusei*; however, for *C. albicans*, the oil was ineffective. Moremi et al. (2021) pointed out the *Croton* species from Southern Africa; *C. gratissimus* Burch., *C. megalobotrys* Müll. Arg., *C. menyhartii* Gürke, *C. pseudopulchellus* Pax, *C. steenkampianus* Gerstner and *C. sylvaticus* Schlldl., showed low inhibitory capacity against *Escherichia coli*. These results suggest the selectivity of essential oils against distinct species of bacteria and fungi.

Furthermore, the antimicrobial activity of CpEO may come from compounds such as Elimicin, Eucalyptol, Bicyclogermacrene, α -terpineol, β -elemene, 4-terpineol and δ -elemene, already mentioned in the topic above. These compounds induce cell instability, causing membrane damage, interference with ATP synthesis, and causing extravasation of intracellular contents [39]. Additionally, it has been reported that genes such as ALS1, ALS3 and HWP1, involved in the biofilm formation process, can be silenced by the action of some plant chemical compounds [40]. This shows the effectiveness of the substances found in oils, which can act as new methods of natural control against microorganisms.

3.3. Mechanisms of Action

3.3.1. Cell membrane integrity

Fluorescence microscopy was used to understand the mechanisms of action of the *CpEO*. The PI cell membrane fluorescent dye became helpful in the identification of damage to yeast cell membranes, and PI binds to the DNA structure and emits red fluorescence. This substance cannot cross the membrane of living cells because they are impermeable, so PI only moves throughout damaged membranes [41].

As expected, the control with DMSO-NaCl showed no fluorescence. However, red fluorescence indicated membrane damage in the planktonic cells of *C. krusei* and *C. parapsilosis* (Figure 1). The same damage on membranes was observed in the biofilm cells in the presence of the *CpEO* (Figure 2). An et al. [42] highlight α -terpineol and terpene-4-ol as compounds that induced damage to the membranes of *Aspergillus niger*, leading to cell death. These same compounds were also identified in *CpEO*.

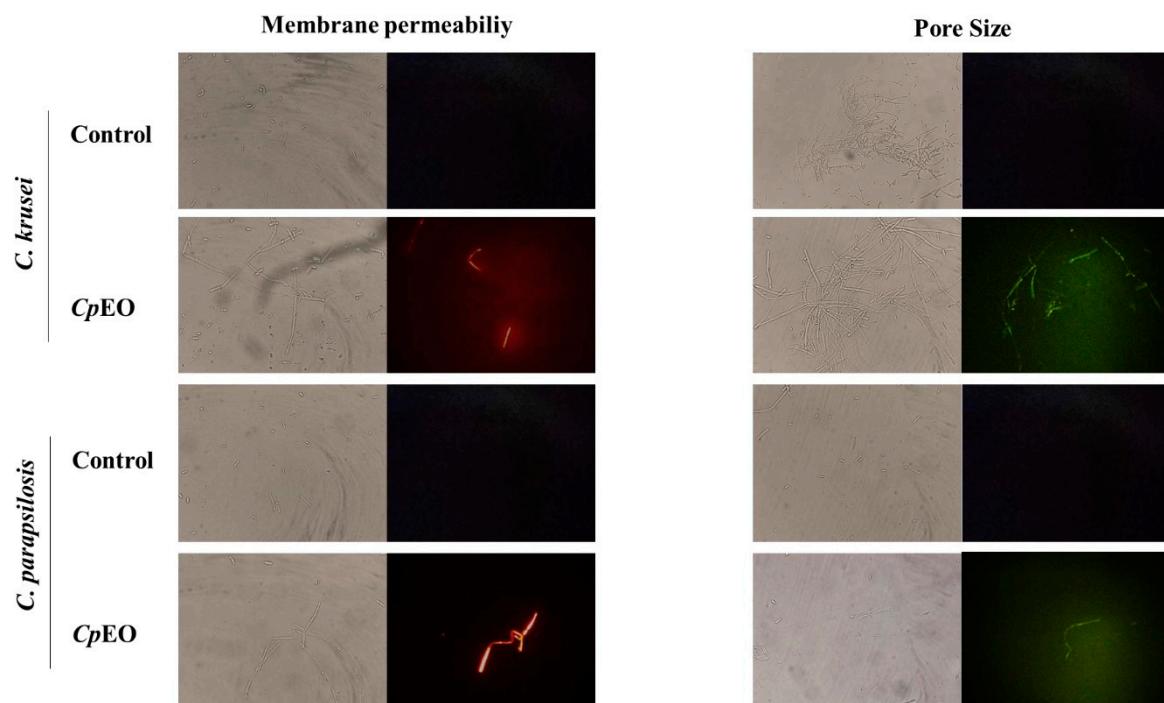


Figure 1. Fluorescence images showing membrane pore formation and their respective size in planktonic cells of *C. krusei* and *C. parapsilosis*. The control was DMSO-NaCl, treated with *CpEO* at 50 μ g mL⁻¹. Membrane pore formation was measured by propidium iodide (PI) uptake assay, and pore size used a 6 kDa dextran-FITC.

It is worth noting that the PI does not reveal the pore size. For this purpose, a new analysis was performed with dextran (6 kDa) in conjunction with FITC (fluorescein isothiocyanate - green fluorescence) in planktonic and biofilm cells (Figures 1 and 2, right panel). The formation of pores in the membrane is revealed by the fluorescence (green), which was stronger in the biofilm, indicating a 6-kDa-sized pore in the cell membranes. The results presented by FITC-Dextran are significant. Although largely used, sometimes PI does not reveal a pore formed. That happens because PI sometimes increases the membrane permeability, which is not necessarily a pore formation. Another problem with PI is due to the size of the pore is too low, allowing the cells to recover [43]. However, both PI and FITC-Dextran assays provided robust results suggesting membrane pore formation.

It is feasible to suggest that the damage caused by *CpEO* to the cell membrane of yeasts is given the presence of components in *CpEO* that were already mentioned in previous topics. Since these

compounds act by interfering with the enzymatic mechanism that supports the structural system of the cell wall, causing morphological damage [44].

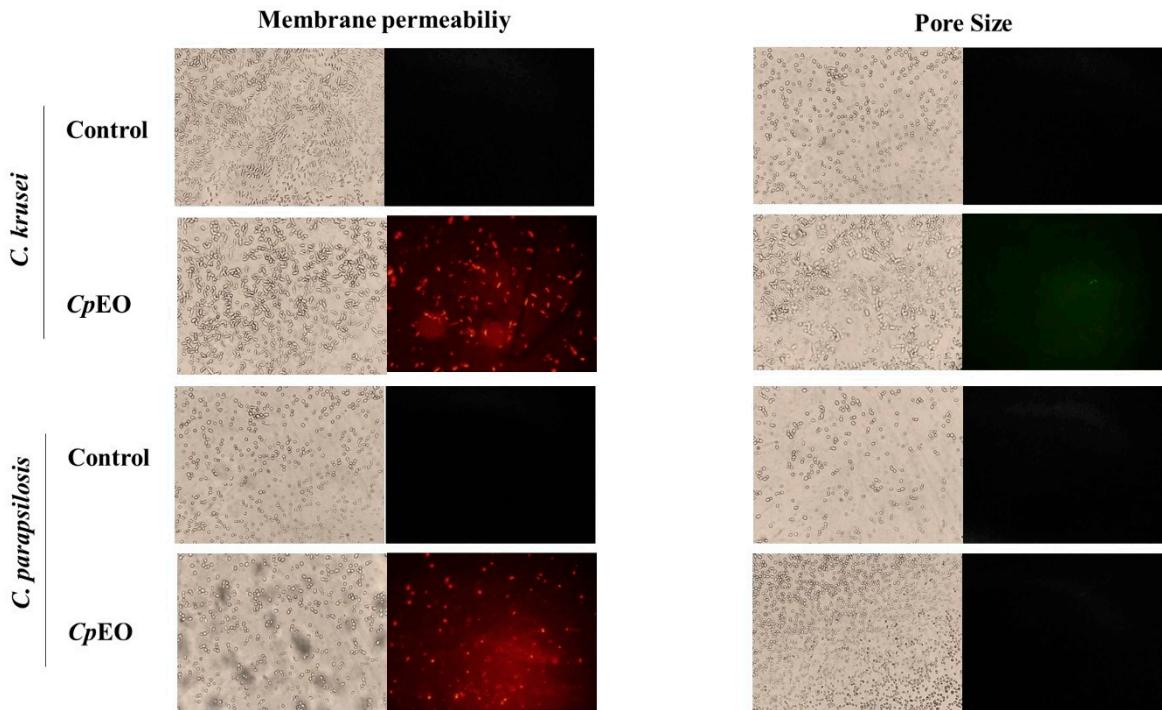


Figure 2. Fluorescence images showing membrane pore formation and their respective size in biofilm cells of *C. krusei* and *C. parapsilosis*. Control used was DMSO-NaCl, treated with CpEO at 50 $\mu\text{g mL}^{-1}$. Membrane pore formation was measured by propidium iodide (PI) uptake assay, and pore size used a 6 kDa dextran-FITC.

3.3.2. ROS Overproduction and Induction of Apoptosis

CpEO induced a slight ROS production in planktonic cells (Figure 3) and biofilm (Figure 4). As expected, DMSO-NaCl did not induce ROS in any treatment. For planktonic cells, CpEO induced ROS accumulation in *C. krusei* and *C. parapsilosis* (Figure 3). However, for a biofilm lifestyle, CpEO was more efficient against *C. krusei* (Figure 4). In the experiment with biofilm, it is trustworthy to mention that in the light field, the number of cells in the treatment with CpEO is smaller than control (Figure 4 – light field). Other reports of ROS overproduction in human pathogenic cells induced by essential oils [16,45,46]. Recently, it was shown that the lavender (*Lavandula angustifolia*) induced ROS accumulation in *Klebsiella pneumoniae* cells, followed by programmed cell death [46].

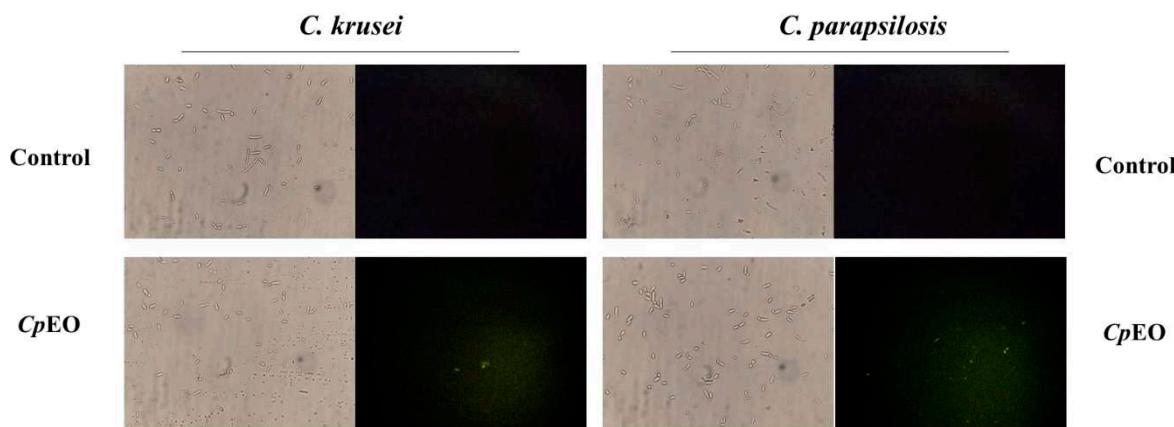


Figure 3. fluorescence images showing overproduction of ROS in planktonic cells of *C. krusei* and *C. parapsilosis*. Control used was DMSO-NaCl, treated with CpEO at 50 $\mu\text{g mL}^{-1}$.

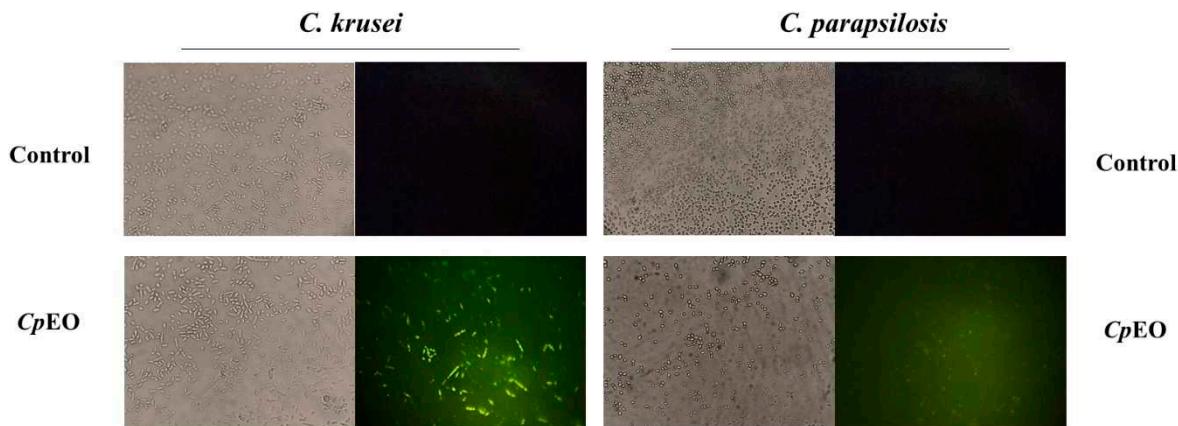


Figure 4. Fluorescence images showing overproduction of ROS in biofilm cells of *C. krusei* and *C. parapsilosis*. Control used was DMSO-NaCl, treated with CpEO at 50 μ g mL⁻¹.

The chemical constituents of essential oils are involved in the formation of ROS by promoting the accumulation of molecules resulting from a disorder in biochemical processes [47]. Different *Cymbopogon martinii* essential oil concentrations stimulated ROS production in *A. flavus* [48]. Thymoquinone, a phytochemical compound found in essential oil, generated oxidative stress in *C. glabrata* [49]. It was recently demonstrated that the essential oil from *C. blanchetianus* leaves, rich in terpene compounds, could induce ROS in planktonic and biofilm cells of *Candida* species [16] hence corroborating with the data found in this research.

ROS are essential molecules for maintaining cellular balance and even establishing biofilms but only at optimal levels [50]. Nonetheless, the levels of ROS must be tightly controlled; if there is an imbalance, their accumulation can cause cell apoptosis by damaging proteins, lipids, carbohydrates, and nucleic acids [51].

Keeping in mind that ROS accumulation induced by CpEO could lead to cell death in *C. krusei* and *C. parapsilosis*, it was performed the analysis of apoptosis in *Candida* cells. Caspase-3/7 activity was observed in all oil-treated cells, less intense in planktonic cells (Figures 5 and 6). As expected, the control (DMSO-NaCl solution) was not induced DNA degradation, as no fluorescence was detected. Thus, CpEO induced DNA damage in *C. krusei* and *C. parapsilosis*. Sun et al. [52] showed that cells treated with eucalyptol and β -cyclocitral activated caspase-3 and caspase-9, proteases involved in the apoptosis process.

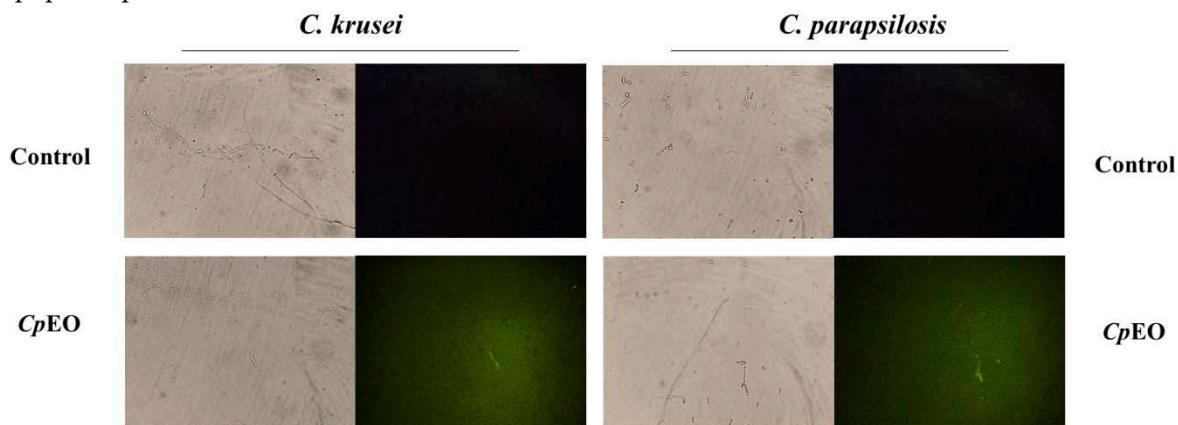


Figure 5. Induction of apoptosis in planktonic cells of *C. krusei* and *C. parapsilosis* using CpEO at concentration 50 μ g mL⁻¹.

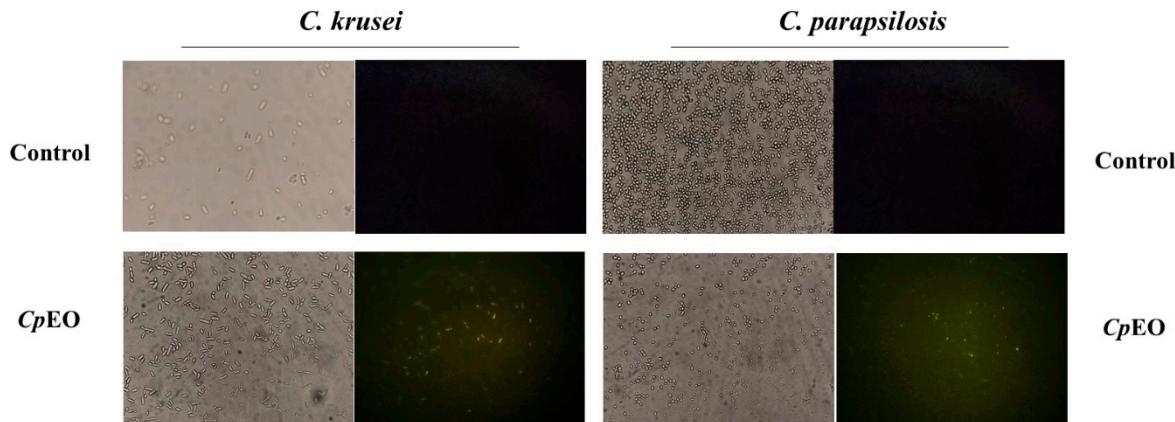


Figure 6. Induction of apoptosis in biofilm cells of *C. krusei* and *C. parapsilosis* using *CpEO* at concentration 50 µg mL⁻¹.

It is known that caspase-3 initiates apoptotic DNA fragmentation and triggers a series of reactions involved in this process [53]. It is worth noting that few studies with essential oils show this type of test in yeast, not describing the mechanism of action of the oil. A recent study revealed by proteomics that limonene induced a higher accumulation of proteins involved in DNA damage and apoptosis [54].

3.4. Scanning Electron Microscopy (SEM)

SEM was used to evaluate the damage caused by *CpEO* in planktonic cells of *C. krusei* and *C. parapsilosis* after all treatments. The images depict that the control cells for both species have healthy anatomy without damage (Figures 7A and 8A). In contrast, the damage is visible in *CpEO*-treated cells. Among them, in cell morphology, deformations in the structure, damaged cell walls, and contorted cells (Figures 7B to F; Figure 8B to F). For both *Candida* species is clear that *CpEO* induced deadly damages. It is possible to see holes in the cell structure, indicating that some parts of the membrane and cell wall are gone.

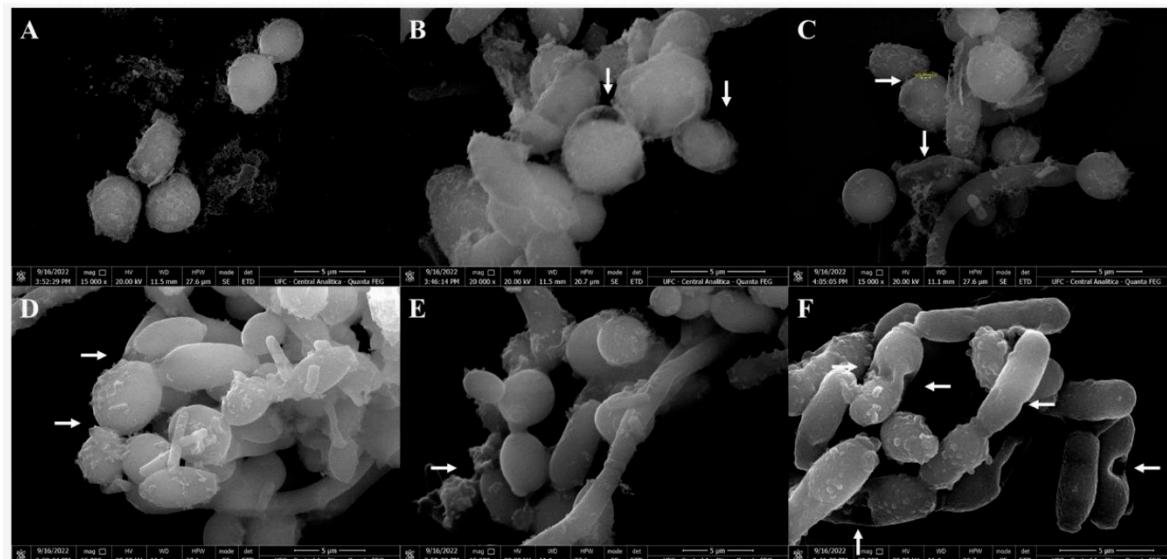


Figure 7. Scanning electron microscopy of *C. krusei* cells treated with *CpEO*. (A) Control *C. krusei* cells treated with DMSO-NaCl solution. (B to F) *C. krusei* cells treated with *CpEO*. White arrows show damage to cell structure.

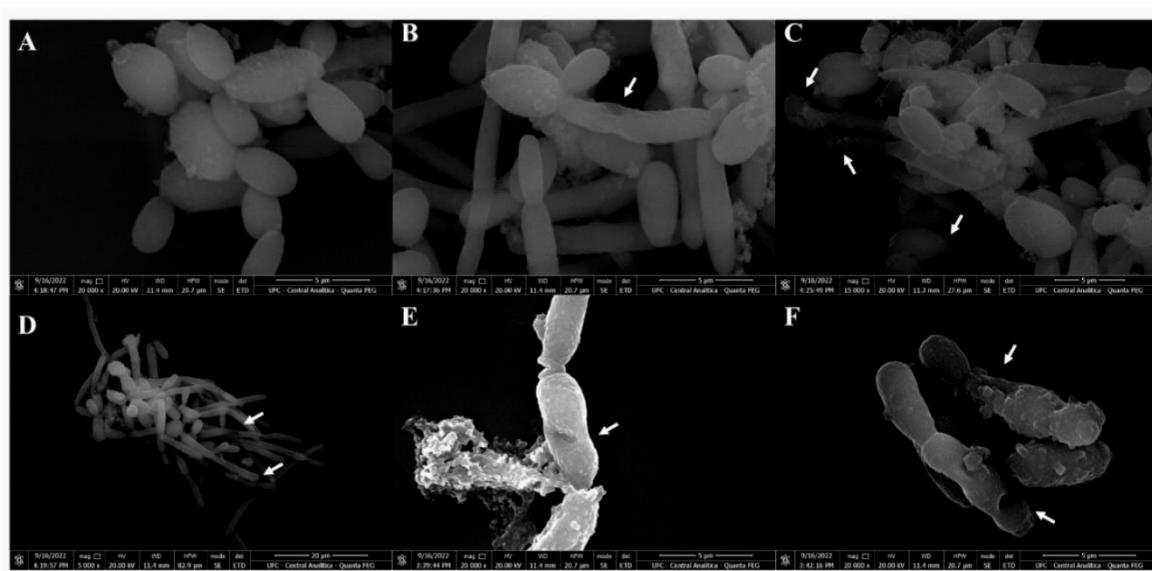


Figure 8. Scanning electron microscopy of *C. parapsilosis* cells treated with CpEO. (A) Control *C. parapsilosis* cells treated with DMSO-NaCl solution. (B to F) *C. parapsilosis* cells treated with CpEO. White arrows show damage to cell structure.

3.5. Hemolytic assay

CpEO did not show hemolytic activity against any human blood type tested (Table 3), even at the concentration of 3 mg mL⁻¹. Thus, CpEO was not toxic to human red blood cells. This result reinforces the pharmacological application of CpEO, as it does not present toxicity risks. This is one of the strong reasons for these substances to be used clinically as drugs [17]. For example, Malveira et al. [16] revealed the oil from *C. blanchetianus* was toxic to red blood cells type O.

Being not harmful to human red blood cells is a critical feature to molecules could be applied in treatment once blood is the vehicle for these molecules. Therefore, CpEO has a great potential to be assessed as source of new antimicrobial molecules.

Table 3. Hemolytic activity of CpEO Against human red Blood cells.

Blood Type	% of hemolysis				
	0.1 % Triton	5% DMSO	<i>CpOE</i> 1 mg mL ⁻¹	<i>CpOE</i> 1.25 mg mL ⁻¹	<i>CpOE</i> 3 mg mL ⁻¹
A	100	0	0	0	0
B	100	0	0	0	0
O	100	0	0	0	0

4. Conclusion

Our research reveals that the essential oil from *Croton pluriglandulosus* Carn.-Torres & Riina leaves represents a possible alternative for the control of human pathogens that already present resistance to conventional drugs offered by the pharmaceutical industry. These molecules of natural origin, have different types of chemical compounds that interfered in the homeostasis of the tested yeast cells. It is possible to suggest and conclude that the oil was efficient against *C. krusei* and *C. parapsilosis*. It revealed membrane and cell wall damage, induced DNA fragmentation, and did not present toxicity to human red blood cells.

Data availability: Data are available under reasonable requirements.

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