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Extract of *Origanum vulgare* L.: Chemical Composition by HPLC-DAD, Antioxidant Activity, Biocompatibility, and Antifungal and Antibiofilm Action

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Abstract

Candida spp. can cause systemic infections with high mortality in immunocompromised patients. Phytotherapy may be an alternative for adjunctive treatment of fungal infections. This study evaluated the phytochemical profile, cytotoxicity, genotoxicity, and antibiofilm activity of the hydroalcoholic extract of *Origanum vulgare* L. against *Candida albicans*, *Candida tropicalis*, and *Candida dubliniensis*. The extraction and quantification (flavonoids and phenols) were performed, and its antioxidant activity (DPPH) and the presence of bioactive compounds were investigated using high-performance liquid chromatography with Diode Array Detection (HPLC-DAD). Cytotoxicity and genotoxicity tests were conducted using HaCat cell lines. Antifungal activity on planktonic cultures was evaluated using the standard (CLSI M27-S4). The analysis of the extract on biofilms was verified with different exposure times. The extract demonstrated the presence of bioactive molecules, and antioxidant activity. The cytotoxicity test showed viability >70%. Genotoxicity revealed the presence of a few micronuclei in some dilutions. The Minimum Fungicidal Concentration was obtained for *C. albicans* and *C. tropicalis*. In the biofilm analysis, there was a reduction of more than 60% in all *Candida* spp. species at the 24 h exposure time. The findings suggest that the *O. vulgare* extract exhibits activity against *Candida* spp. and shows biocompatibility in human keratinocytes.

Keywords: biofilm; *Candida* spp.; cytotoxicity; *Origanum vulgare*; phytotherapy

Academic Editors: Marcello Locatelli, Zdenek Wimmer and Michal Jurasek

Received: 24 March 2026
Revised: 27 April 2026
Accepted: 9 July 2026
Published: 11 July 2026

1. Introduction

The increasing incidence of antifungal-resistant *Candida* spp. infections is a global health concern [1]. As a commensal microorganism, *Candida* spp. is commonly found in the gastrointestinal tract, skin, oral mucosa, and female genitourinary tract. However, under immunosuppressive conditions, it becomes an opportunistic pathogen, particularly in Intensive Care Unit (ICU) settings, where cross-contamination poses a significant risk [2,3].

Candida spp. is a major cause of nosocomial fungal bloodstream infections, with species such as *Candida albicans*, *Candida tropicalis*, *Candida glabrata* (*Nakaseomyces glabratus*), *Candida parapsilosis*, and *Candida krusei* (*Pichia kudriavzevii*) frequently isolated from clinical samples. The emergence of *Candida auris* (*Clavispora auris*), first identified in Japan in

2009, has increased concerns due to its high mortality rate and resistance to multiple drugs, particularly among immunocompromised patients with COVID-19 [2,3]. This species exhibits resistance to most antifungal agents, with echinocandins being the primary treatment option for this fungus [4,5].

These yeasts are classified into *C. albicans* and non-albicans species due to the fact that *C. albicans* is the main species of the genus that causes infections in debilitated individuals [6]. Although not pathogenic under immunological homeostasis, non-albicans species can cause infections, with some strains exhibiting antifungal resistance [2,7,8]. Pathogenicity is associated with morphological transitions from yeasts to pseudohyphae and hyphae, which allow tissue invasion under favorable environmental conditions, such as high CO_2 levels and immunosuppression [9,10].

Nosocomial *Candida* spp. infections are frequently linked to biofilm formation on medical devices, including catheters and endotracheal tubes. Biofilms protect fungal cells from physical, chemical, and biological stresses due to their thick extracellular matrix, which hinders the absorption of any antimicrobial particles [7,11,12]. The oral cavity, part of the gastrointestinal tract, is particularly susceptible to biofilm formation due to its diverse microbiota, which, when unbalanced, facilitates opportunistic *Candida* spp. infections [13,14].

Biofilms formed by *Candida* spp. increase resistance by producing hydrolytic enzymes that degrade antifungals such as amphotericin B, fluconazole, and caspofungin, or by employing efflux pump mechanisms within the membrane of ergosterol, glucan, and mannan [13]. Because yeast is a eukaryotic organism, the development of antifungal agents that selectively target fungal cells without harming human cells remains a challenge, as antifungal drugs have a high cytotoxicity margin. Ergosterol, the fungal counterpart of human cholesterol, serves as a primary antifungal target [14–16].

Natural compounds with potential antifungal and biocompatibility are being explored in various parts of the world. *O. vulgare* L. is an aromatic plant distributed throughout Asia, already used for the treatment of respiratory tract diseases. Its phytochemical components include, rosmarinic acid, apigenin-7-glucoside, luteolin-7–glucoside, ursolic acid and phenolic compounds (phenolic acids and flavonoids) [17]. Extracts produced from certain parts of *O. vulgare* L. demonstrate significant antioxidant activity and biocompatibility on human keratinocytes [18].

Plant extracts such as rosemary, lavender, ginger, pomegranate, turmeric, cinnamon and thyme have demonstrated inhibitory effects against *Candida* spp. [14,19,20].

In vitro studies with *Rosmarinus officinalis* L. (rosemary) have shown significant biofilm reduction in *Candida* spp. and bacterial pathogens [21]. Similarly, extracts of *Curcuma longa* L. (turmeric), *Rosmarinus officinalis* L. (rosemary), and *Thymus vulgaris* L. (thyme) have increased macrophage activity against *Streptococcus mutans* [22]. Other promising antifungal sources include *Origanum vulgare* L. (oregano), which exhibit antimicrobial, antioxidant, and antifungal properties [23–25]. The phenolic compounds in *O. vulgare* L. demonstrate antifungal activity, with carvacrol identified as a key bioactive component, although its cytotoxicity should be carefully evaluated [26–28].

Therefore, the objective of the study was to evaluate the chemical composition of the hydroalcoholic extract of *Origanum vulgare* L., its antifungal activity in planktonic cultures and biofilms of *Candida albicans*, *Candida tropicalis* and *Candida dubliniensis*, and to evaluate its cytotoxicity and genotoxicity.

2. Materials and Methods

2.1. Phytochemical Analyses

2.1.1. Preparation of *O. vulgare* L. Extract and Quantification of Soluble Solids Content

The hydroalcoholic extract of *O. vulgare* L. (CIA Natural Saúde, Caçapava, SP, Brazil) was prepared using 30 g of dried, moisture-free leaves. The extraction vehicle consisted of a combination of absolute ethanol (Contemporary Chemical Dynamics Ltd., Indaiatuba, SP, Brazil) and ultrapure water obtained from a Milli–Q® system (EtOH:H_2O, 50:50). The extraction was performed at a ratio of 30 g of plant material per 100 mL of the vehicle, with an extraction time of 48 h, protected from light and under stirring (70 rpm, COLEMAN, TS-200 = VDRL Shaker, Curitiba, PR, Brazil). The extract was then filtered in two sequential steps: solid residues were removed using a paper filter with microholes (J. Prolab, São José dos Pinhais, PR, Brazil), followed by sterilization through a 0.22 µm membrane filter. The final extract was stored in an amber bottle and protected from light [29].

For the quantification of solids and soluble content, three empty 50 mL beakers were individually weighed, and their masses recorded. Subsequently, 5 mL aliquots of the *O. vulgare* L. hydroalcoholic extract were transferred into each beaker (triplicate) and subjected to drying at 80 °C for 24 h, until complete evaporation of the solvent. After drying, the soluble solids content in the extract was determined according to the equation below:

$$\% \text{ soluble solids (m/v)} = (m - b) \times 100/Vo, \quad (1)$$

where % m/v is the content of soluble solids; (m − b) is the mass of the beaker with the extract (m) minus the mass of the beaker without the extract (b); Vo is the volume of extract used for the analysis of soluble solids (5 mL).

2.1.2. Determination of Total Phenol Content of the Plant Extract

A stock solution (10 mg/mL) was prepared by transferring a 1 mL aliquot of the *O. vulgare* L. extract into a 100 mL volumetric flask, dissolved with 5 mL of ethanol (Contemporary Chemical Dynamics Ltd., Indaiatuba, SP, Brazil), and gradually bringing the volume to the mark with distilled water under stirring. All subsequent steps were performed in triplicate. A 200 µL aliquot was transferred into a 10 mL volumetric flask (1:50) containing about 5 mL of distilled water, followed by the addition of 800 µL of Folin–Ciocalteau reagent (Contemporary Chemical Dynamics Ltd., Indaiatuba, SP, Brazil). The mixture was stirred for 10 s, and after 8 min, 1.2 mL of 20% sodium carbonate-tartrate buffer solution was introduced. The volume was then adjusted to just below the meniscus with water, and the solution was incubated in a water bath at 20 °C for 2 h. After incubation, the volume was brought up to the final mark at room temperature, stirred for 5 s, and absorbance was read at 760 nm using a spectrophotometer (B582, Micronal, São Paulo, Brazil). The concentration of total phenols expressed as gallic acid was determined using the standard curve equation [29].

2.1.3. Determination of Total Flavonoid Content of the Plant Extract

To determine the total flavonoid content, a stock solution was prepared by transferring 100 µL of the *O. vulgare* L. extract into a 10 mL volumetric flask, followed by the addition of methanol (P.A. Labsynth, Diadema, SP, Brazil) to the meniscus. All steps were performed in triplicate. A 200 µL aliquot was taken from the stock solution and transferred into a 10 mL flask containing about 5 mL of methanol, followed by the addiction of 200 µL of aluminum chloride ($AlCl_3$) (Contemporary Chemical Dynamics Ltd., Indaiatuba, SP, Brazil). The volume was then adjusted to approximately 5 mL with methanol, and the mixture was stirred and incubated in a water bath at 20 °C for 30 min. After incubation, the volume

was adjusted, and the absorbance was read at 425 nm using a spectrophotometer (B582, Micronal, São Paulo, Brazil). The concentration of total flavonoids expressed as quercetin was determined using the equation of the straight line from the standard curve [29].

2.1.4. Evaluation of the *O. vulgare* L. Extract by High—Performance Liquid Chromatography (HPLC–DAD)

The extract of *O. vulgare* L. was analyzed by High—Performance Liquid Chromatography (HPLC—Merck Hitachi—Lachrom L7000 series, Darmstadt, Germany) with a diode grating detector (DAD), using a C18 Lichrochart—Lichrospher Merck (Darmstadt, Germany) 100 RP—18—5 µm, 12.5 cm column for separation. The sample was filtered using a membrane filter (PES—22 µm, Inforlab, Teresina, Brazil) attached to a syringe to separate the extract from any type of contaminant. Each chromatographic run lasted 45 min with an injection volume of 20 µL.

The chromatographic conditions were established as follows: the mobile phase consisted of formic acid (PA, Merck) diluted to 5% in ultrapure water (solvent A) and chromatographic methanol—Merck (solvent B), at a flow rate of 1 mL/min, using a linear gradient:

0 to 4 min—100% solvent A

5 to 20 min—80% solvent A and 20% solvent B

21 to 33 min—70% solvent A and 30% solvent B

34 to 45 min—100% solvent B

Detection was performed out at wavelengths of 280 nm and 320 nm. The chemical composition was evaluated by comparison with a spectral library [29].

2.2. Evaluation of the Free Radical Scavenging Activity of the Plant Extract

This method is based on the reduction in the 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH, Sigma–Aldrich, St. Louis, MI, USA) in absolute ethyl alcohol solution (Contemporary Chemical Dynamics Ltd., Indaiatuba, Brazil), which presents maximum absorption in the range of 515–528 nm. The samples were prepared at different dilutions based on the total soluble solids content (SSC) of the *O. vulgare* L. hydroalcoholic extract, previously determined. The volume of extract required to prepare dilutions at 1% (*v*/*v*) and 0.01% (*v*/*v*) was calculated. The corresponding aliquot of the 1% (*v*/*v*) solution was transferred into a 10 mL volumetric flask, and the volume was completed with ethanol. In 11 test tubes, numbered 0 to 10, 1000, 960, 920, 880, 840, 800, 760, 720, 680, 640, or 600 µL of absolute alcohol were added, respectively. Subsequently, 0, 40, 80, 120, 160, 200, 240, 280, 320, 360, or 400 µL of 0.01% extract were added on top of the ethanol. Tube 0 served as the control (100% DPPH without the extract) and 1000 µL of DPPH was added into it. After 60 s, DPPH was added to the remaining tubes at 1 min intervals, following the numerical sequence. After 30 min from the addition of DPPH to tube 0, absorbance was measured using a spectrophotometer (Micronal B582, São Paulo, Brazil) at 517 nm. All readings were performed in triplicate, and the absorbance values were used to obtain the percentage of remaining DPPH versus the concentration of the plant extract (µg/mL). The EC50 (concentration that eliminates 50% of free radicals) was determined using a linear regression (% remaining DPPH versus extract concentration in µg/mL) [29].

2.3. Biocompatibility Analysis of the Plant Extract

2.3.1. Evaluation of the Cytotoxicity

The cytotoxic potential of *Origanum vulgare* L. extract was evaluated in human keratinocyte cell lines (HaCaT) obtained from the Rio de Janeiro Cell Bank—Paul Ehrlich Technical-Scientific Association (APABCAM—RJ). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM—LGC Biotecnologia, Cotia, Brazil), with a high glucose concentration, supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island,

NY, USA) and maintained in cell culture flasks (TPP, Trasadingen, Switzerland), incubated in an oven at 37 °C, with atmospheric humidity and 5% CO_2. For cell disaggregation, the cell monolayer was detached from the flask surface using trypsin. Cell counting was performed in a Neubauer chamber (Tiefe Depth Profundeur, 0.100 mm, 0.0025 mm^2), and the cells were then sent to their respective tests once the required quantity was reached.

Cytotoxicity of the extract was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma—Aldrich, Jurubatuba, Brazil). colorimetric assay, based on cellular enzymatic activity. For this purpose, cell cultures were transferred to 96—well microplates (Corning Incorporated Costar, REF 3599, Corning, NY, USA) (TPP) at a concentration of 2×10^4 viable cells per well in 200 µL of DMEM supplemented with 10% FBS. Plates were then incubated at 37 °C with 5% CO_2 for 24 h to promote cell adhesion.

Cells were then exposed to *O. vulgare* L. extract at varying concentrations for 1 h and 24 h, with the extract diluted in DMEM supplemented with 10% FBS. The control group received DMEM alone. Following exposure, cells were incubated with MTT solution (0.5 mg/mL in DMEM + 10% FBS) for 4 h. The solution was subsequently discarded, and 100 µL/well of dimethyl sulfoxide (DMSO—Sigma) was added. After 10 min of incubation under shaking, absorbance of the wells was read in a microplate reader at a wavelength of 570 nm. The optical densities (OD) values were converted into percentage of cell viability to determine the cytotoxic potential of the extract [30].

2.3.2. Evaluation of Genotoxicity

The micronucleus test followed the OECD 2016 guideline (TG487). HaCaT cells (5×10^5) were cultured in 24–well plates for 24 h at 37 °C with 5% CO_2 (Sanyo CO_2 Incubator, Panasonic Group, Chicago, IL, USA). The cells were then exposed to DMEM supplemented with 10% FBS and ethyl methanesulfonate (EMS; 5 mM, positive control) for 24 h. Cytochalasin B (6 µg/mL) was added for a further 24 h to inhibit cytokinesis. The cells were subjected to hypotonic shock, followed by fixation in methanol:acetic acid (3:1) and DAPI staining. Micronuclei were analyzed by fluorescence microscopy (400X, Leica Microsystems, São Paulo, SP, Brazil) in 2000 cells/well in two independent experiments. Micronuclei have been defined as DNA structures in the cytoplasm, distinct from the main nucleus, with a size $\leq 1/3$ of the nuclear area [30].

2.4. Evaluation of Antifungal and Antibiofilm Activity of the Plant Extract

2.4.1. Fungal Inoculum Preparations

Reference strains (ATCC—American Type Culture Collection) of *C. tropicalis* (ATCC 13803), *C. dubliniensis* (ATCC MYA 646), and *C. albicans* (ATCC 18804), provided by the Aerobic and Anaerobic Laboratory (LANA) of ICT–UNESP, were used.

Yeasts of the genus *Candida* spp. were cultivated in an incubator suitable for (37 °C, 24 h) in BHI (Brain Heart Infusion Broth, Kasvi, Pinhais, PR, Brazil). After the medium became turbid, it was seeded onto Sabouraud–Dextrose agar (SD—Himedia, Mumbai, MH, India) (37 °C/24 h). Planktonic cultures were standardized at 10^6 CFU/mL and biofilms at 10^7 CFU/mL with regulation at 530 nm in a spectrophotometer (B582, Micronal, São Paulo, Brazil).

2.4.2. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *O. vulgare* L. Extract

The broth microdilution method was performed following CLSI M27—S4 guidelines with modifications. The *Candida* spp. inoculum was standardized at 530 nm (OD 0.284) to a concentration of 1×10^6 CFU/mL. Serial 1:2 dilutions of the extract were prepared in Mueller Hinton broth (Kasvi, Brazil) in 96—well microplates (Kasvi K12—096, São José do Pinhais, Brazil). 100 µL aliquots of each fungal suspension were added to all wells. After

24 h at 37 °C, the minimum inhibitory concentration (MIC) would be identified as the last well without visible turbidity. All assays were performed in duplicate. For the minimum fungicidal concentration (MFC), aliquots from all wells were plated on Sabouraud Dextrose agar and incubated for 48 h at 37 °C. Following incubation, colony growth was evaluated, and the MFC was defined as the lowest concentration at which no colony growth was detected. A vehicle control group (EtOH:H_2O, 50:50) was included to assess possible interferences in the extract's activity.

2.4.3. Evaluation of the Antibiofilm Activity of the Plant Extract

The analysis of biofilms followed Meccatti et al. [31]. *Candida* spp. suspensions (10^7 CFU/mL) were standardized, and 200 µL/wells were added to microplates. After 90 min (pre—adhesion period) at 37 °C, non-adherent cells were removed, and Brain Heart Infusion (BHI) broth was added. Biofilms were formed over 48 h with broth replacement at 24 h. The formed biofilms were then exposed to concentrations of plant extract (based on MFC results) for 1 or 24 h. Negative control: culture medium; positive control: 0.06% chlorhexidine (n = 8) was applied. Following exposure to the treatments, the broth was discarded, and the wells were rinsed with sterile saline solution (0.9% NaCl) prior to cell viability analysis using the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma—Aldrich, Jurubatuba, Brazil). Subsequently, 100 µL per well of the MTT solution (0.5 mg/mL) was added, and the plate was incubated in the dark at 37 °C for 1 h. After incubation, the MTT solution was removed and replaced with 100 µL of Dimethyl Sulfoxide (DMSO) (Synth, Diadema, SP, Brazil). The plate was then incubated again at 37 °C for 10 min, followed by constant agitation on a shaker for an additional 10 min. Optical densities (OD) values were then measured using a microplate reader at 570 nm, and the obtained values were converted into a percentage of microorganism viability reduction using the following formula:

$$\%\ \text{viability reductin} \ = \ 100 \ - \ \left(\frac{OD\ treatment\ average}{OD\ negative\ control\ average} \times \ 100 \right)$$

where

$$\text{Average} = \left(\frac{sum\ of\ measurements}{10} \right)$$

performed individually for the treatment groups, positive control, and negative control.

2.5. Statistical Analysis

For statistical analysis, normally distributed data were evaluated by ANOVA followed by Tukey's test, while non-normally distributed data were assessed using the Kruskal–Wallis test followed Dunn's post hoc test. All analyses were performed using GraphPad Prism 5.0, with a significance level of 5%.

3. Results

3.1. Chemical Profile

3.1.1. Soluble Solids Content

The quantification of the soluble solids content (SSC) of the hydroalcoholic extract is shown in Table 1 The SSC indicates the number of soluble substances that were extracted, thus determining, in the triplicate test, the actual concentration of the *O. vulgare* L. extract.

Table 1. Soluble solids, phenolic and flavonoid content of *O. vulgare* L. extract.

Plant Part	Extraction Solvent	Parameter	Value (mg/mL)	SD
Leaves (*O. vulgare*)	EtOH:H_2O (50:50)	SSC	3.1527	0.0190
		Phenols	0.888	±0.127
		FLV	0.076	±0.030

Legend: EtOH—absolute ethyl alcohol; H_2O—distilled water; SSC—soluble solids content; FLV—flavonoid content; SD—standard deviation of three determinations.

3.1.2. Determination of Total Phenols in the Hydroalcoholic Extract of *O. vulgare* L.

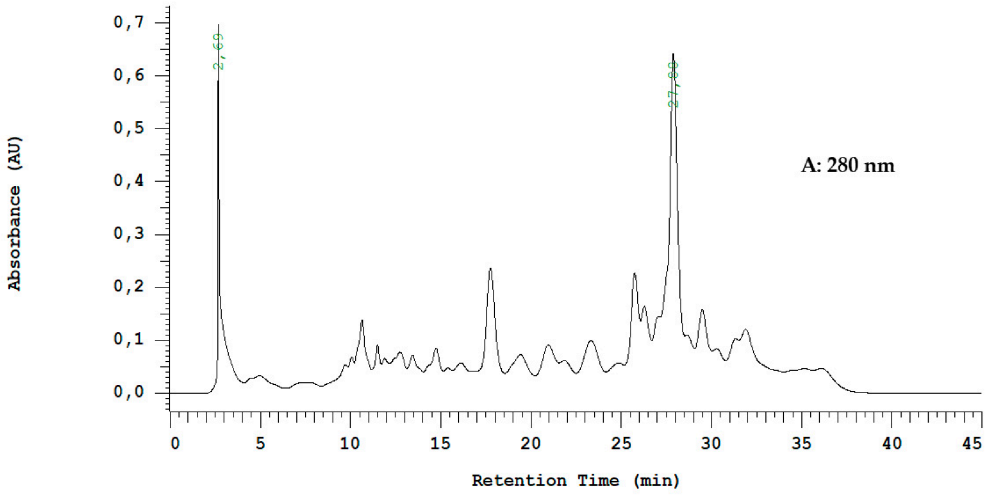
Based on the construction of the gallic acid standard curve, the total phenols of the Hydroalcoholic Extract of *O. vulgare* (OHE) at 20 °C were quantified: the total phenol content was 0.888 mg/mL, as shown in Table 1.

3.1.3. Determination of the Total Flavonoid Content of the Hydroalcoholic Extract of *O. vulgare* L.

Based on the quercetin standard curve, the amount of total flavonoids in the hydroalcoholic extract (Table 1) was analyzed. For the hydroalcoholic extract, the flavonoid concentration was 0.076 mg/mL, with a standard deviation of 0.030.

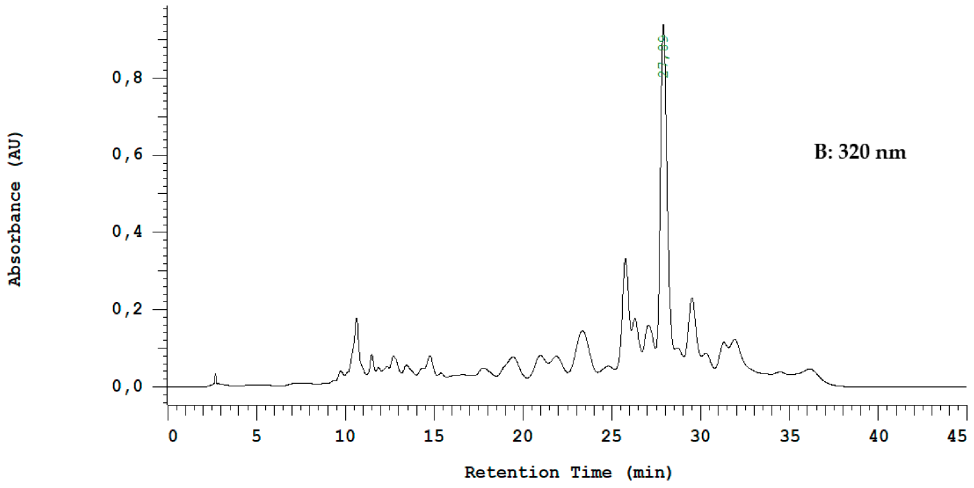
3.1.4. Chemical Composition by HPLC

HPLC analysis identified important compounds in the hydroalcoholic extract of *O. vulgare* (OHE) at wavelengths of 280 nm and 320 nm. The chromatograms (Figure 1A,B) revealed peaks corresponding to carvacrol, caffeoylquinic acid, chlorogenic acid, vitexin, isovitexin, and rosmarinic acid. At 280 nm (Figure 1A), retention times included gallic acid (2 min), carvacrol (~5 min), apigenin (~14 min), and cinnamic acid derivative ~27 min. At 320 nm (Figure 2B), retention times were: chlorogenic acid (~6 min), caffeoylquinic acid (~10 min), rosmarinic acid (~28 min), and vitexin (~31 min).



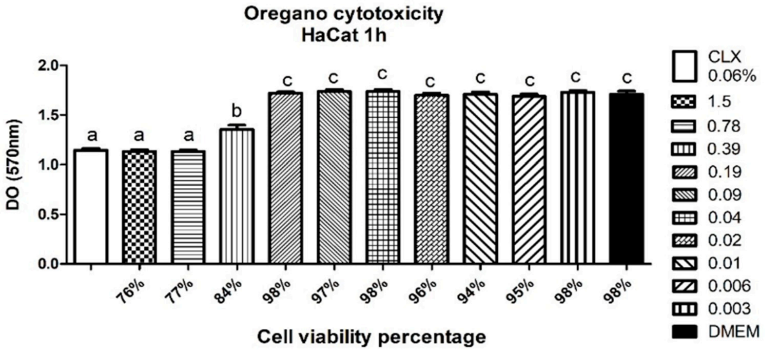
(**A**)

Figure 1. *Cont.*

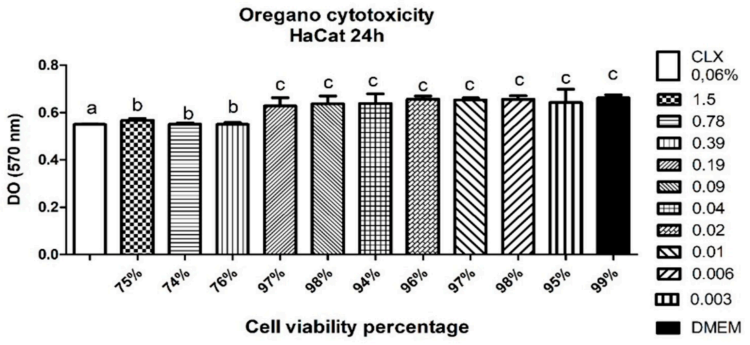


(**B**)

Figure 1. (**A**) chromatogram of hydroalcoholic extract of oregano at 280 nm (**A**), with retention time of gallic acid ~2 min, carvacrol ~5 min, apigenin ~14 min, cinnamic acid derivative ~27 min. (**B**) chromatogram of hydroalcoholic extract of *O. vulgare* L. (OHE) at 320 nm, with retention time of chlorogenic acid ~6 min, caffeoylquinic acid ~10 min, rosmarinic acid ~28 min, vitexin ~31 min.



(**A**)



(**B**)

Figure 2. (**A**) Percentage of cell viability after exposure to the hydroalcoholic extract of *O. vulgare* L (mg/mL) at 1 h. (**B**) Percentage of cell viability after exposure to the hydroalcoholic extract of *O. vulgare* L. (mg/mL) for 24 h. DO—Optical density; OHE—Hydroalcoholic extract of *O.vulgare* L.; CLX—Chlorhexidine 0.06%; Vehicle—EtOH:H_2O (50:50); Control—DMEM; the letters "a", "b", "c" indicate the statistically significant similarity or difference between the groups ($p < 0.0001$).

3.2. Antioxidant Activity of the Hydroalcoholic Extract of *O. vulgare* L.

The antioxidant activity of the hydroalcoholic extract of *O. vulgare* L. is shown in Table 2. The concentration that eliminates 50% of free radicals (EC50) was 35.64 µg/mL, with a standard deviation of 0.46 µg/mL.

Table 2. Evaluation of antioxidant activity.

Plant Part	Extraction Solvent	EC50 (µg/mL)
Leaves (*O. vulgare* L.)	EtOH:H_2O (50:50)	35.64 ± 0.46

Legend: EtOH—absolute ethyl alcohol; H_2O—distilled water; EC50—concentration that eliminates 50% of free radicals.

3.3. Biocompatibility of the Plant Extract

3.3.1. Cytotoxicity of *O. vulgare* L. Extract in Human Keratinocytes (HaCat)

After 1 h of contact with the plant extract, cell viability remained above 70% in all conditions analyzed. Although the groups treated with the highest concentrations of the extract showed statistically significant differences compared to the negative control (DMEM medium), some concentrations did not show significant differences, with the overall average percentage of cell viability reaching approximately 80% (Figure 2A).

After 24 h of treatment, cell viability remained above 70% in all conditions tested (Figure 2B). The highest concentrations, however, showed statistically significant differences compared to the negative control (DMEM medium). In contrast, some concentrations showed no significant changes, with the overall average cell viability remaining around 80%.

3.3.2. Genotoxicity of *O. vulgare* L. Extract

Genotoxicity analysis of the hydroalcoholic extract of *O. vulgare* in human keratinocytes (HaCaT) revealed significant genotoxic effects, particularly at higher concentrations. Compared to the negative control (DMEM), there was a notable increase in micronuclei per cell, indicating potential genetic damage after 24 h of exposure at the highest concentrations.

At 1.5 mg/mL, 79.2% of the cells exhibited micronuclei, while 0.78 mg/mL and 0.39 mg/mL showed 23.2% and 18.2%, respectively. Fluorescence microscopy identified chromosomal fragmentation and genetic rearrangements, especially at higher concentrations. As shown in Figure 3, 0.39 mg/mL was the safest concentration to maintain genetic integrity.

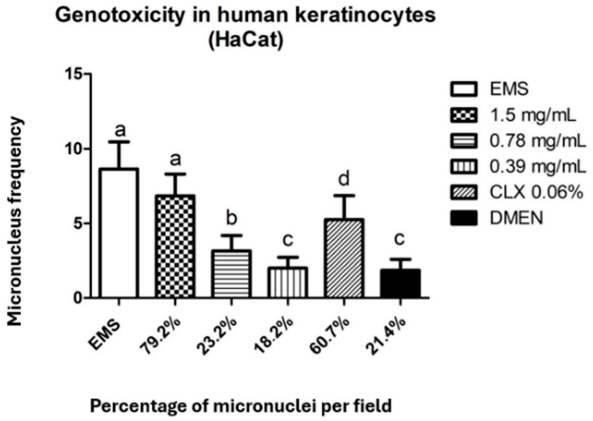


Figure 3. Analysis of genotoxic effects in human keratinocytes (HaCat). CLX—chlorhexidine 0.06%; Control—DMEN; the letters "a", "b", "c", and "d" indicate the statistically significant similarity or difference between the groups ($p < 0.0001$).

3.4. Antifungal and Antibiofilm Activity of the Plant Extract

3.4.1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the *O. vulgare* L. Extract

The MIC values were identical to those of the MFC, confirming the fungicidal activity of the hydroalcoholic extract of *O. vulgare* (OHE). The MFC was observed against *C. albicans* (0.19 mg/mL) and *C. tropicalis* (0.78 mg/mL), but not against *C. dubliniensis*, suggesting an MFC above 0.78 mg/mL. The total soluble solids content was 3.15 mg/mL. The assay was performed in duplicate, and the MFC values are presented in Table 3.

Table 3. Determination of MFCs in yeasts.

Soluble Solids Content of OHE	***Candida albicans* (ATCC 18804)**	***Candida tropicalis* (ATCC 13803)**	***Candida dubliniensis* (MYA 646)**
3.15 mg/mL	MFC 0.19 mg/mL	MFC 0.78 mg/mL	-

Legend: OHE = Hydroalcoholic Extract of *O. vulgare* L.

3.4.2. Evaluation of the Antibiofilm Action of *O. vulgare* L. Extract

The concentrations used for biofilm analyses were selected based on the total soluble solids content (SSC) of the plant extract (3.15 mg/mL—pure extract):

- 1.5 mg/mL (concentration equivalent to ½ of SSC—for *C. dubliniensis*: the strain that was not affected by the extract in planktonic form);
- 0.78 mg/mL (concentration equivalent to ¼ of SSC—for *C. tropicalis* corresponds to MFC and for *C. albicans* corresponds to 4x MFC);
- 0.39 mg/mL (concentration equivalent to $^{1}/_{8}$ of SSC—for *C. tropicalis* corresponds to ½ MFC and for *C. albicans* corresponds to 2x MFC);
- 0.19 mg/mL (concentration equivalent to $^{1}/_{16}$ of SSC—for *C. albicans* corresponds to MFC).

Among the experimental groups, there were also the Control (−) (negative control—culture medium) and CHX (positive control—0.06% chlorhexidine digluconate).

Antibiofilm Action of the Extract on *C. albicans*

For *C. albicans* biofilm, after 1 h of treatment with hydroalcoholic of *O. vulgare* L. extract (OHE), a concentration of 0.78 mg/mL reduced biofilm formation by 52.2%, while 0.39 mg/mL and 0.19 mg/mL achieved 43.3% and 44.2% eradication, respectively. A significant difference ($p < 0.05$) was observed between the 0.78 mg/mL and chlorhexidine groups when compared to the negative control (Figure 4A).

After 24 h, a concentration of 0.78 mg/mL eradicated 73% of the biofilm, while 0.39 mg/mL and 0.19 mg/mL achieved 69.7% and 55.1% eradication, respectively (Figure 4B). The highest OHE concentrations (0.78 mg/mL and 0.39 mg/mL) demonstrated similar efficacy to 0.06% chlorhexidine ($p > 0.05$). All experimental groups showed a statistically significant difference when compared to the negative control ($p < 0.05$).

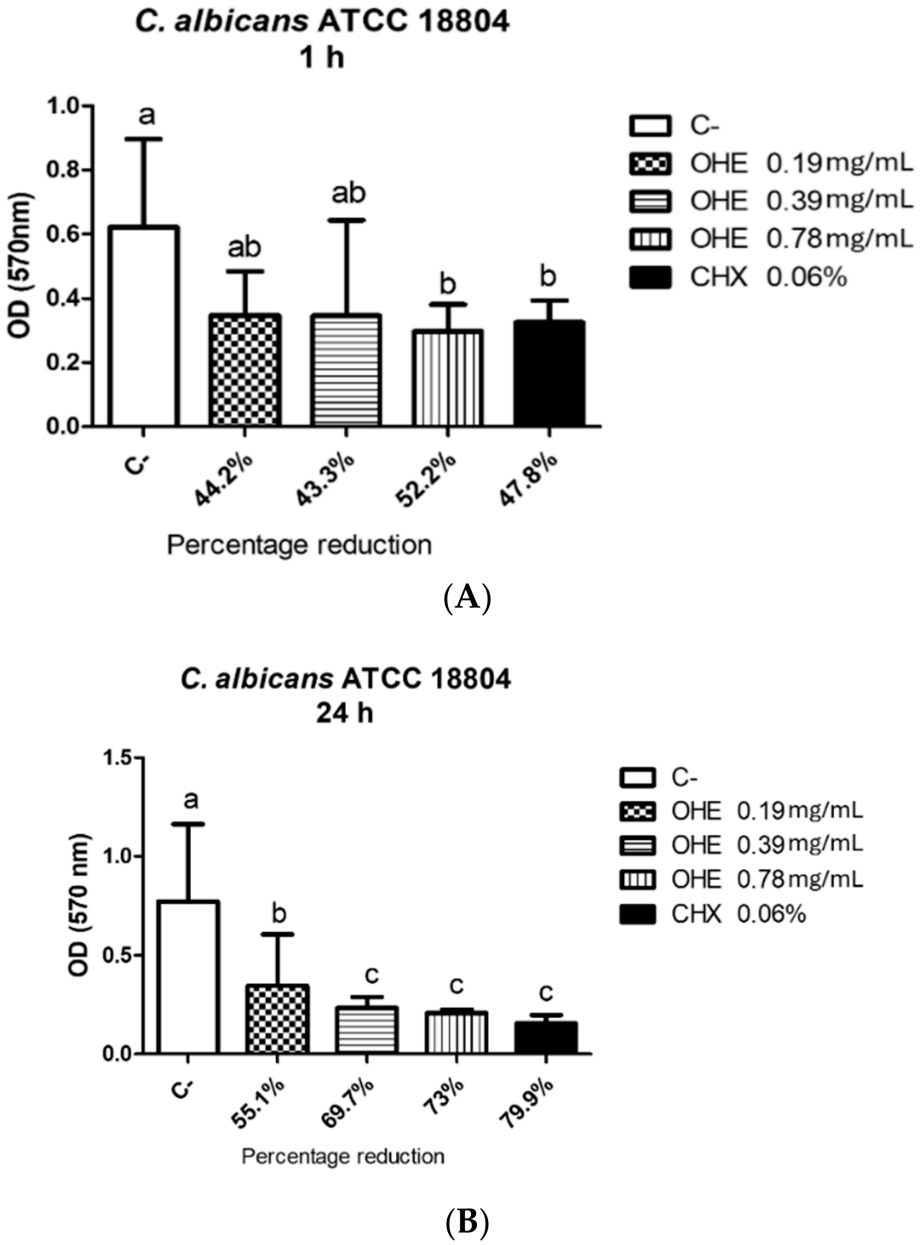


Figure 4. (**A**) Evaluation of the cell viability of the monotypic *Candida albicans* biofilm after application of the plant extract (1 h). (**B**) Evaluation of the cell viability of the monotypic *Candida albicans* biofilm after application of the plant extract (24 h). OD—Optical density; OHE—hydroalcoholic extract of *O. vulgare* L.; CHX—chlorhexidine 0.06%. The letters "a", "b", and "c" indicate statistically significant similarities or differences between the groups ($p < 0.05$).

Antibiofilm Action of the Extract on *C. tropicalis*

For *C. tropicalis* (ATCC 13803), after 1 h, OHE at 0.78 mg/mL eradicated 66.5% of the biofilm, while 0.39 mg/mL achieved a reduction of 47.1% (Figure 5A). The 0.78 mg/mL group showed statistical similarity to chlorhexidine ($p > 0.05$). After 24 h, 0.78 mg/mL eradicated 60.7% of the biofilm ($p < 0.05$ vs. control) and 0.39 mg/mL reduced the biofilm by 40.6% (Figure 5B).

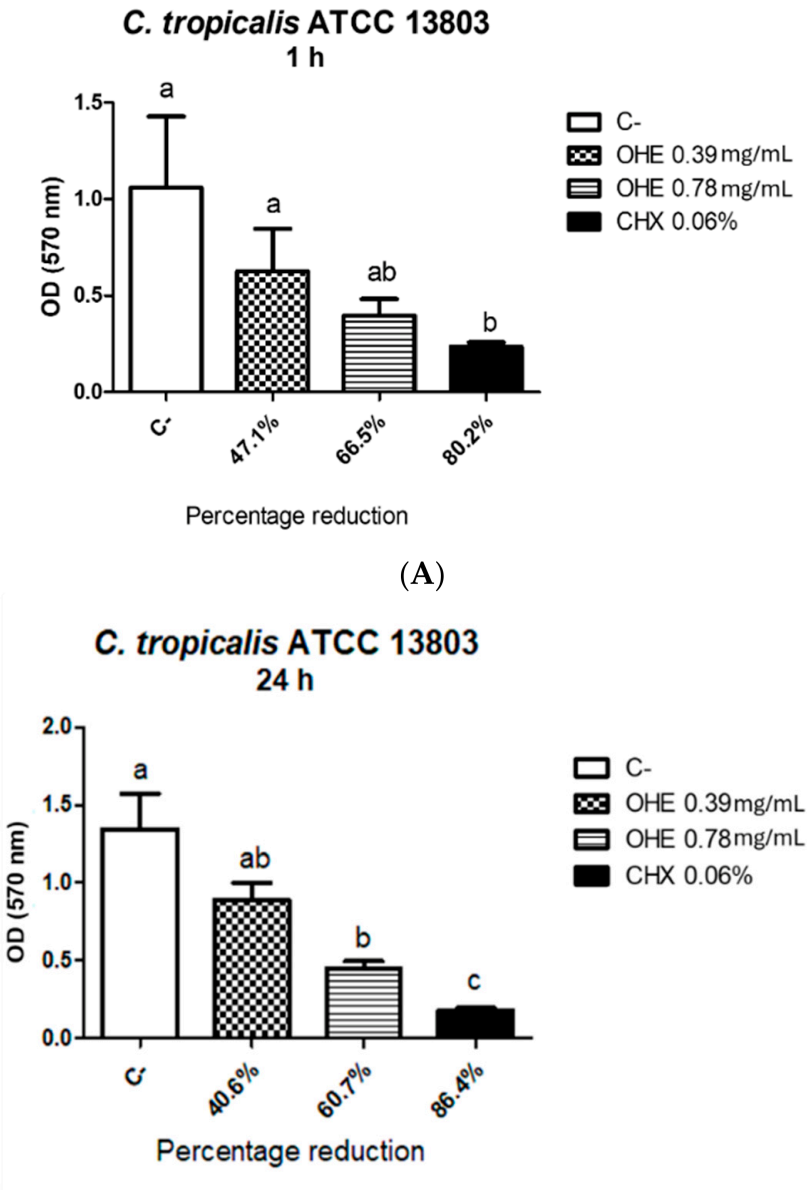


Figure 5. (**A**) Evaluation of the cell viability of the monotypic *Candida tropicalis* biofilm after application of the plant extract (1 h). (**B**) Evaluation of the cell viability of the monotypic *Candida tropicalis* biofilm after application of the plant extract (24 h). OD—Optical density; OHE—hydroalcoholic extract of *O. vulgare* L.; CHX—chlorhexidine 0.06%. The letters "a", "b", and "c" indicate the statistically significant similarity or difference between the groups ($p < 0.05$).

Antibiofilm Action of the Extract on *C. dubliniensis*

For *C. dubliniensis* (MYA 646), after 1 h, OHE at 1.5 mg/mL and 0.78 mg/mL eliminated 17.9% and 21.2% of the biofilm, respectively (Figure 6A). The group treated with the 0.78 mg/mL concentration showed a statistically significant difference compared to the negative control ($p < 0.05$). After 24 h of treatment, OHE at a concentration of 1.5 mg/mL eradicated 71.8% of the *C. dubliniensis* biofilm, and the 0.78 mg/mL concentration resulted in 70.9% eradication, with both concentrations showing a statistically significant difference when compared to the negative control ($p < 0.05$) (Figure 6B).

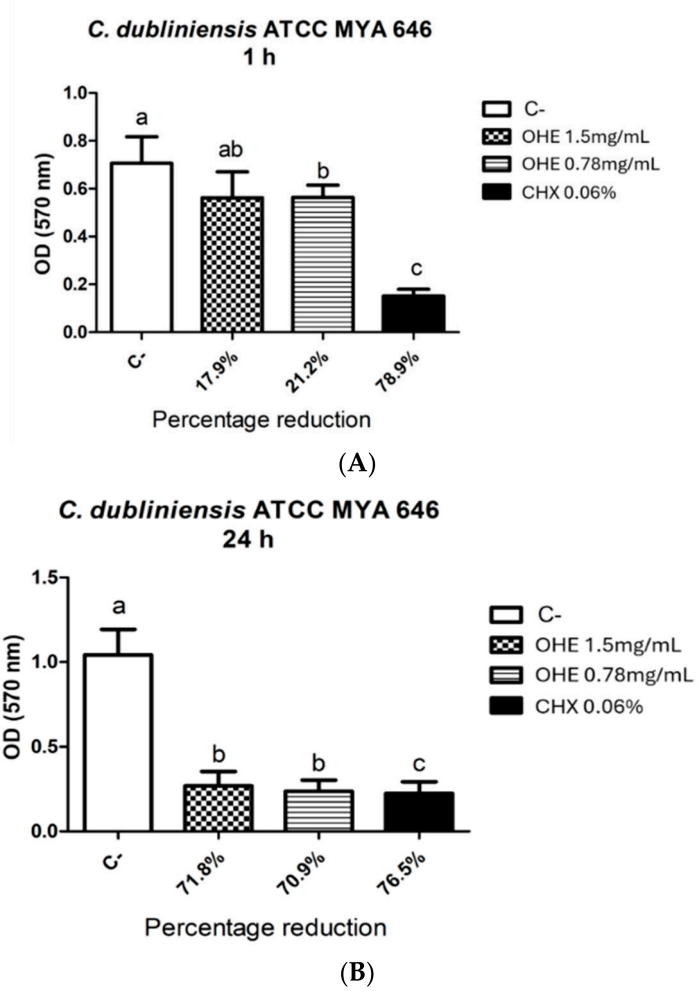


Figure 6. (**A**) Evaluation of the cell viability of the monotypic *Candida dubliniensis* biofilm after application of the plant extract (1 h). (**B**) Evaluation of the cell viability of the monotypic *Candida dubliniensis* biofilm after application of the plant extract (24 h). OD—Optical density; OHE—hydroalcoholic extract of *O. vulgare* L.; CHX—chlorhexidine 0.06%. The letters "a", "b", "c" indicate the statistically significant similarity or difference between the groups ($p < 0.05$).

4. Discussion

Due to the growing need for new antimicrobial molecules as alternatives for treating opportunistic fungal infections, this study analyzed the biological activities of the hydroalcoholic extract of *O. vulgare* L. (OHE) [MS1] in planktonic cultures and monotypic biofilms in vitro, along with phytochemical analyses and biocompatibility in human keratinocytes.

O. vulgare L. has been extensively studied in phytotherapy, mainly through its essential oils and ethanolic extracts [16,32,33]. However, little is known about the chemical composition and antimicrobial properties of its hydroalcoholic extract. Liaqat et al. [16] highlighted that the chemical composition of *O. vulgare* L. varies depending on the geographical location. In this study, phenols (0.888 mg/mL) and total flavonoids (0.076 mg/mL) were identified and quantified. Touaitia et al. [34] describe that the essential oil of *O. vulgare* is rich in phenolic monoterpenes and sesquiterpenes and exhibits broad-spectrum antimicrobial activity. These findings align with previous studies, such as Samira et al. [35] and Kosakowska et al. [26], which reported concentrations of flavonoids and phenols in oregano essential oils. Carvacrol, one of the main antimicrobial compounds, was detected in our hydroalcoholic extract using high-performance liquid chromatography (HPLC), corroborating previous findings on *O. vulgare* L. [36].

The variations observed in chemical compositions between studies can be attributed to the use of different extraction methods and solvent choices, which impact efficiency [37,38]. Our HPLC analysis detected rosmarinic acid, carvacrol, chlorogenic acid, vitexin, isovitexin (320 nm), gallic acid, and apigenin (280 nm), confirming that the extract of *O. vulgare* L. contains many bioactive compounds [39,40].

Regarding antioxidant activity, the hydroalcoholic extract of *O. vulgare* L. demonstrated an EC_{50} of 35.64 µg/mL using the DPPH free radical scavenging method. Previous studies reported EC_{50} values of 0.55 µg/mL for oregano oil [41] and 0.77 µg/mL for ethanolic extracts [42]. Despite the existing literature on the antioxidant properties of oregano, our study is among the first to evaluate a hydroalcoholic extract using this method.

The antimicrobial and antibiofilm potential of OHE was evaluated against *Candida* spp. *C. albicans* exhibited a minimum fungicidal concentration (MFC) of 0.19 mg/mL, while *C. Tropicalis* had an MFC of 0.78 mg/mL. The literature reports the antimicrobial effects of *O. vulgare* L. against various bacteria and *Candida* spp. [14,43]. However, studies on hydroalcoholic extracts remain scarce. *C. dubliniensis* exhibited resistance to OHE (> 0.78 mg/mL), which may be related to the overexpression of the efflux pump and target ERG11 enzymatic alterations [44].

The antimicrobial activity of oregano has been demonstrated against multidrug-resistant bacteria, including *Escherichia coli* and *Klebsiella pneumoniae* [45], as well as *Streptococcus salivarius* (MIC: 3.0 mg/mL, MBC: 1.5 mg/mL) [32] and *Streptococcus pyogenes* [15]. Our study confirms the antimicrobial potential of hydroalcoholic extracts, which are less explored. The average percentage of biofilm eradication after 24 h of treatment with OHE was 65% for *C. albicans*, *C. tropicalis*, and *C. dubliniensis*. Previous studies reported eradication rates of 80-87% for *Staphylococcus aureus* [46] and efficacy against oral pathogens (*Fusobacterium nucleatum, Porphyromonas gingivalis* and *Streptococcus mutans*) [47].

Cytotoxicity analysis in human keratinocytes (HaCat) revealed more than 85% cell viability at 0.39 mg/mL after 1 and 24 h, while higher concentrations (1.5 mg/mL and 0.78 mg/mL) were cytotoxic. This suggests that 0.39 mg/mL is a safe concentration for potential formulations. Stojanović et al. [48] reported similar findings, showing that high concentrations of oregano oil induced cytotoxic effects in hepatocytes and renal cells in rats.

Genotoxicity analysis showed that 1.5 mg/mL and 0.75 mg/mL of OHE induced micronucleus formation, while 0.39 mg/mL did not. These alterations suggest possible interference with DNA repair mechanisms and chromosomal integrity, aligning with previous research indicating that high concentrations of plant extracts may have genotoxic potential [48].

Although *O. vulgare* L. has well-documented therapeutic properties, studies on its hydroalcoholic extract remain limited. This study demonstrated significant antioxidant and antifungal activity against *Candida* spp. in both planktonic cultures and biofilms, without cytotoxicity or genotoxicity at a concentration of 0.39 mg/mL. However, more studies and evaluations using clinical strains are needed to explore their application in integrative medical and dental treatments.

5. Conclusions

The hydroalcoholic extract of *Origanum vulgare* L. presented bioactive compounds in significant quantities, notably flavonoids and phenols, resulting in significant antioxidant activity. It demonstrated antifungal action against planktonic cultures of *Candida albicans* and *Candida tropicalis*. In *Candida* spp. biofilms, the most effective treatment time was 24 h, with an average eradication rate of 65%, similar to the gold standard of asepsis (chlorhexidine) in some situations. Furthermore, the extract did not show cytotoxic or genotoxic effects on human HaCat keratinocytes at a concentration of 0.39 mg/mL.

Author Contributions: Conceptualization, G.M.d.C., M.C.M., L.D.d.O. and V.M.M.-D.; methodology, G.M.d.C., L.D.d.O., M.C.M. and V.M.M.-D.; formal analysis and investigation, G.M.d.C., T.M.F.C., R.T.d.M., L.S.d.C. and P.d.S.A.; writing—original draft preparation, G.M.d.C. and G.T.T.; writing—review and editing, G.T.T., M.C.M., L.D.d.O. and V.M.M.-D.; supervision, M.C.M., L.D.d.O. and V.M.M.-D.; project administration, V.M.M.-D. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001, especially for the master's scholarship number: 88887.939882/2024-00. We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for their financial support through PhD scholarship number: 140181/2023-7 and to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the Scientific Initiation Grant (process 2023/16016-5).

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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