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In Vitro Evaluation of Selected Essential Oils as Possible Antifungal and Antibiofilm Agents

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Abstract

Microbial biofilms are organized consortiums of microorganisms in the self-produced matrix, characterized by increased resistance to antimicrobial agents. *Candida albicans* belongs to the regular human microbiota, but it could be highly pathogenic. Essential oils (EOs) are widely distributed secondary metabolites, proven for various biological activities. The main goal of this investigation was to evaluate the antifungal and antibiofilm properties of EOs from *Citrus limon* (L.) Osbeck, *C. reticulata* Blanco, *Nigella sativa* L., and *Foeniculum vulgare* Mill. against *C. albicans*. Antifungal activity was evaluated through the disk diffusion method, followed by the determination of the minimum inhibitory (MIC) and minimum fungicidal concentration (MFC). Antibiofilm assays were implemented through the tissue culture plate method and determination of the biofilm inhibition. Zones of inhibition were detectable for all tested EOs, with the greatest activity of *N. sativa* (28.30±1.50 mm to 39.30±1.10 mm). MIC values ranged from 62.50 µg/ml (*N. sativa*) to 125 µg/ml (*C. limon*), and 250 µg/ml (*C. reticulata* and *F. vulgare*). All tested EOs performed an impact on the biofilm-forming capacity of tested yeast. The antibiofilm activity was species-specific and concentration-dependent. The highest antibiofilm activity was recorded for *F. vulgare*. Obtained results suggest that investigated EOs possess antifungal and antibiofilm potential.

Keywords: essential oils, antifungal and antibiofilm, *Citrus limon* (L.) Osbeck, *Citrus reticulata* Blanco, *Nigella sativa* L., *Foeniculum vulgare* Mill.

Резюме

Микробните биофилми са организирани консорциуми от микроорганизми в самопроизвеждаща се матрица, характеризиращи се с повишена резистентност към антимикробни средства. *Candida albicans* принадлежи към обичайната човешка микробиота, но може да бъде и силно патогенна. Етеричните масла (ЕМ) са широко разпространени вторични метаболити с доказани различни биологични активности. Основната цел на това изследване е да се оценят противогъбните и антибиотичните свойства на EOs от *Citrus limon* (L.) Osbeck, *C. reticulata* Blanco, *Nigella sativa* L. и *Foeniculum vulgare* Mill. срещу *C. albicans*. Антифунгалната активност е определена чрез дисково-дифузионен метод, последван от установяване на минималната инхибираща (MIC) и минималната фунгицидна концентрация (MFC). Антибиофилмовите анализи са осъществени чрез метода на плаките за тъканни култури и определяне на инхибирането на биофилма. Зони на инхибиране са установени за всички тествани ЕО, като най-голяма активност имаше това от *N. sativa* (28.30±1.50 mm до 39.30±1.10 mm). Стойностите на MIC варират от 62.50 µg/ml (*N. sativa*) до 125 µg/ml (*C. limon*) и 250 µg/ml (*C. reticulata* и *F. vulgare*). Всички тествани ЕО оказват въздействие върху способността на тестваните дрожди да образуват биофилми. Антибиофилмовата активност е видово специфична и зависима от концентрацията. Най-висока антибиофилна активност е отчетена при използването на ЕО от *F. vulgare*. Получените резултати предполагат, че изследваните EOs притежават антифунгален и антибиофилмов потенциал.

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Introduction

The development of antimicrobial drugs such as antibiotics and antimycotics had a tremendous impact on modern medicine, where it could be discussed easier and prolonged life, reduced childhood mortality, and increased life expectancy. Nevertheless, antimicrobial resistance is considered one of the major global health challenges of the 21st century. *Candida albicans* is a member of the regular human microbiota, but under specific circumstances, it can cause possibly fatal infections (Gulati and Nobile, 2016). This species is characterized by its high biofilm-forming ability, resistance to antifungal drugs as well as the expression of various virulence factors (Cavalheiro and Teixeira, 2018). Almost every *Candida* species differs in some specific elements of the biofilm formation, and in the case of *C. albicans* there are some crucial steps including the adhesion, germ tube formation, hyphal elongation, accumulation of extracellular matrix (ECM), and dispersion of cells from the biofilm. These cells express higher pathogenicity, filamentation, adhesion, and prominent capacity for further formation of the biofilm (Eix and Nett, 2020). Challenges in the treatment of *C. albicans* infections, particularly biofilm-involving ones, are reflected through the different aspects of the biofilm formation, specifics in the morphology, ECM diversity, and unpredictable antifungal resistance capacity (Golia *et al*., 2012). Antimycotic drug resistance, avoiding the host immune response joint with the high toxicity of some antifungal drugs, emphasizes the need for the identification of novel effective, acceptable, and affordable compounds against *C. albicans*.

Essential oils (EOs) are defined as plant secondary metabolites that represent complex mixtures mainly consisting of 20 to 60 constituents. Several hundred EOs are commercialized and widely utilized in everyday use (Wińska *et al*., 2019). Many biological activities such as antiseptic, antimicrobial, antioxidant, and insecticidal are previously reported for EOs, but one of the crucial and most investigated aspects of their biological activity refers to the antimicrobial effects. EOs and their components could be considered naturally occurring antimicrobial agents.

Citrus limon (L.) Osbeck, lemon (Rutaceae) is worldwide used for various purposes, especially in cooking, but investigations also proved its biological activities such as anticancer, antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, antiallergic, hepatoregenerative, anti-diabetic, and anti-obesity. Lemon has also shown an effect on the nervous, cardiovascular, respiratory, and skeletal systems (Klimek-Szczykutowicz *et al*., 2020). *Citrus reticulata* Blanco (Rutaceae), mandarine possesses numerous biological activities such as: antimicrobial, anticancer, antioxidant, neuropharmacological, and hepatoprotective. Furthermore, it has antigenotoxic, and antihypercholesterolemic effects (Musara *et al*., 2020). *Nigella sativa* L. (Ranunculaceae), frequently known as black cumin is a worldwide distributed plant whose pharmacological significance is reflected in the ability to act as an antimicrobial and antioxidative agent, besides the other curative effects on human health (Tabassum *et al*., 2018). *Foeniculum vulgare* Mill. (Apiaceae), fennel is a seasonal medicinal plant, grown by humans in nearly every region. This is a popular cooking ingredient because of its great aromaticity and spiciness. Pharmacological studies suggest that fennel could be used in the treatment of many diseases since its different bioactive effects (Tripathi *et al*., 2012). Plant species included in this study were investigated earlier for their antifungal properties, but there are no available data regarding the antibiofilm properties for all of them. The main goal of this investigation was to evaluate the antifungal and antibiofilm capacity of essential oils from *C. limon*, *C. reticulata*, *N. sativa*, and *F. vulgare*.

Materials and methods

Essential oils

In this study, essential oils of *C. lemon* (L.) Osbeck, *C. reticulata* Blanco, *N. sativa* L., and *F. vulgare* Mill. (Dea Flores d.o.o., Rijeka, Croatia) were used. Stock solutions of the test substances were prepared in dimethyl sulfoxide ≥99% (DMSO) (Sigma-Aldrich, St. Louis, MO) and kept at room temperature in the dark.

Investigated species

In this research, *C. albicans* (Robin) Berkhout (ATCC 10231) was used. The reference strain was purchased from the American Type Culture Collection, ATCC (Manassas, Virginia, USA). The fungal suspension is made by adding 1.0 ml of sterile distilled water in the ampoules with a lyophilized pellet. Suspension is after transferred back into the test tube with distilled water and left for 2 hours at room temperature. After mixing, several drops are used to inoculate the growth medium. According to the supplier's recommendation, the inoculum was incubated and viability was observed after 1-2 days of incubation. Selected strain is frequently used for pharmacological purposes, agricultural research,

investigation of antimicrobial resistance, drug development, etc.

Determination of the antifungal activity

The antifungal activity of investigated essential oils was evaluated by using undiluted EO, as well as different concentrations of EO (750, 500, and 250 µg/ml) dissolved in 0.1% DMSO. For *in vitro* screening, the disk diffusion method was applied. The fungal strain was cultured in Sabouraud Glucose Agar (Fluka Biochemica; Buchs, Switzerland). According to the National Committee for Clinical Laboratory Standards (2015), inoculums were adjusted in sterile saline solution to the final density of 0.5 McFarland standard (~1.5 x 10^8 CFU/ml). The fungal suspension was spread over the growth medium plates with the sterile cotton swab. The amount of 10 µl of each concentration was impregnated into the paper disk. In total, six disks were placed on one 150 mm plate, including the positive and negative control. As the positive control, antimycotic Nystatin (100 units; Oxoid Ltd., England) was used, while DMSO served as the negative control. After the application of essential oil plates were incubated at 35±2°C for 24 h. Antifungal activity was evaluated by the diameter (mm) of the obtained inhibition zones. All tests were performed in three replications, and the mean value ± standard deviation (STDEV) was taken for further analysis.

Minimum inhibitory (MIC) and minimum fungicidal concentration (MFC)

MIC values were determined through the broth microdilution method. Overnight cultures of the fungal cells were adjusted to the turbidity of 0.5 McFarland standard (1-5 × 10^6 CFU/ml) in Sabouraud Glucose Broth, SGB (Sigma-Aldrich, St. Louis, MO). The final inoculum suspension (1×10^3 CFU/ml) was prepared by dilution with SGB. Afterward, each well of the 96-well microtiter plate was inoculated with 100 µl of yeast suspension and an equal volume of two-fold dilutions of EO (ranging from 1000 to 1.95 µg/ml), which resulted in 5.0 × 10^2 cells/ml (NCCLS, 2002). DMSO was used as a solvent in which EOs were dissolved to the final concentration of 1000 µg/ml. SGB inoculated by fungal culture was used as a positive control, while the SGB medium with DMSO was taken as the negative control. Microtiter plates were incubated overnight at 35±1°C in the incubator with natural convection (Binder BD 53E2). After incubation, results were read on a microplate reader (Biochrom EZ Read 400) at the wavelength of 595 nm. These experiments were performed in four replications. After reading of MIC results, the content of the well described as the MIC and the content from the two surrounding wells were replated on a sterile Sabouraud Glucose Agar, SGA (Sigma-Aldrich, St. Louis, MO) in order to determine the minimum fungicidal concentration (MFC). Plates were incubated overnight at 37°C. This experiment was performed in triplicate.

Antibiofilm potential of selected EOs

For the evaluation of the antibiofilm potential of tested EOs, the tissue culture plate (TCP) method in 96-well plates (Merritt *et al*., 2005) was used, with the tryptic soy broth (Sigma-Aldrich, St. Louis, MO) as the dilution medium. The investigated EOs were two-fold diluted in TSB up to the end concentration of 1.95 µg/ml. An amount of 100 µl of dilutions was added to each well, followed by inoculation with 10 µl of the tested yeast strain. The biofilm formation was determined through the adherence of cells only in the presence of TSB. The content of the plates was decanted after overnight incubation, while plates were washed in Phosphate-Buffered Saline, PBS (Sigma-Aldrich, St. Louis, MO) and stained with 0.1% crystal violet for 10 minutes. Upon the dye, 96% ethanol was added to the wells, and the results were read on the microplate reader (Biochrom EZ Read 400) at 595 nm. The experiment was done in quadruplets, and the results are given as mean ± STDEV. The category of the biofilm was determined in accordance with Stepanović *et al*. (2007) and by using the Biofilm Classifier Software ver 1.1. The optical density cut-off value (ODc) was calculated as three standard deviations above the mean OD of the negative control, while the biofilm categories were determined as: OD ≤ ODc: non-adherent (NA), ODc < OD ≤ 2×ODc: weakly adherent (W), 2×ODc < OD ≤ ×4xODc: moderately adherent (M), and 4×ODc < OD: strongly adherent (S). The percentage of biofilm inhibition was calculated according to (Jadhav *et al*., 2013):

$$\% = \frac{OD_{595\ nm}\ \text{of experimental well with the extract}}{OD_{595\ nm}\ \text{of control well without the extract}} \times 100$$

Statistical analysis

Descriptive statistical parameters (mean values and standard deviation) and the percentage of biofilm inhibition were calculated using Microsoft Office 2019 Excel (Microsoft Corporation, USA). Data were further analyzed by using one-way ANOVA and *post-hoc* Fisher's LSD test (STATIS-

TICA 10; StatSoft. Inc.) at the significance level of $p<0.05$.

Results

Inhibition zones

The results of the antifungal activity of tested EOs obtained through the disk diffusion method are presented in Table 1. Inhibition zones (IZ) are achieved with all tested EOs in all concentrations. In comparison to the control (IZ=21.30±0.60 mm), the EO of the *N. sativa* showed greater activity with the inhibition zones ranging from 28.30±1.50 mm to 39.30±1.10 mm (Table 1). Statistical significance between the results is indicated in Table 1.

Table 1. Antifungal activity of tested essential oils with obtained inhibition zones

Tested plant	Concentration	Inhibition zone (mm)
	Undiluted essential oil	14.00±1.40[a, b]
C. limon[1]	750 µg/ml	16.50±0.70[a]
	500 µg/ml	16.50±0.70[b, c]
	250 µg/ml	14.00±1.40[c]
	Undiluted essential oil	17.70±1.20[a]
C. reticulata[2]	750 µg/ml	20.70±1.50[a]
	500 µg/ml	10.30±0.60[a]
	250 µg/ml	7.20±0.45[a]
	Undiluted essential oil	39.30±1.10[a]
N. sativa[1, 2, 3]	750 µg/ml	38.60±2.80
	500 µg/ml	28.30±2.90
	250 µg/ml	28.30±1.50[a]
	Undiluted essential oil	9.30±0.50[a, b]
F. vulgare[1, 2, 3]	750 µg/ml	8.00±0.00
	500 µg/ml	7.30±1.00[a, c]
	250 µg/ml	7.00±1.00[b, c]
Nysatin[1, 2, 3]	100 IU	21.30±0.60

Results are mean ± STDEV; NI = No inhibition; DMSO = NI; Numbers in superscript indicate statistically significant differences in the activity of tested EOs and the control, after performing *post-hoc* Fisher's LSD test. Values with the same number differ significantly at $p<0.05$. Letters in superscript indicate statistically significant differences in the activity of particular concentrations of tested EO after performing post-hoc Fisher's LSD test. Values with the same letter differ significantly at $p<0.05$.

MIC and MFC

The obtained MIC values of EOs ranged from 62.50 µg/ml (*N. sativa*) to 250 µg/ml (*C. reticulata* and *F. vulgare*), while Nystatin showed MIC at 31.25 µg/ml. According to this, the highest antifungal activity is performed by the *N. sativa* essential oil (Table 2). The MFC was not recorded for tested EOs since there was a presence of fungal growth after replating on a sterile medium. The MFC of Nystatin was 62.50 µg/ml.

Impact on the biofilm formation

According to the abovementioned classification, the investigated strain of *C. albicans* performed a strong ability to form biofilm. Examined EOs caused changes in the biofilm-forming capacity of *C. albicans* as follows. *C. limon* EO did not change the biofilm-forming category in subinhibitory concentrations, but a certain amount of inhibition was noted. The highest percentage of biofilm inhibition for this sample is recorded at 7.81 µg/ml (27.37%).

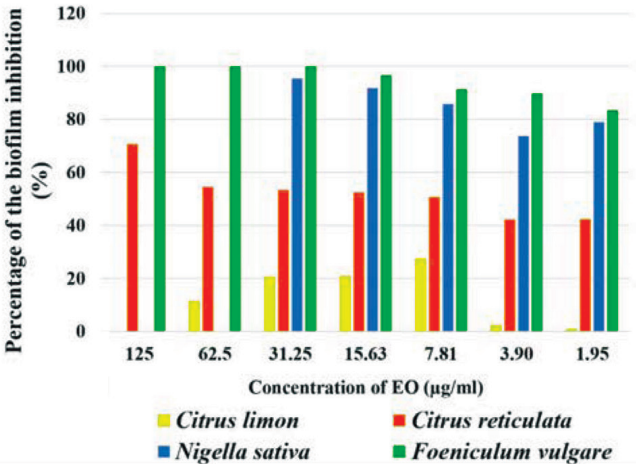

Fig. 1. Inhibition of *C. albicans* biofilm (%) by investigated essential oils

The activity of *C. reticulata* EO is illustrated by decreasing the biofilm-forming category from strong- to moderately adherent in the range of 125-7.81 µg/ml, and inhibition of the biofilm formation in the amount of 50.68-70.68%. The first two subinhibitory concentrations of *N. sativa* EO (31.25 µg/ml and 15.63 µg/ml) have caused the elimination of *C. albicans* biofilm with inhibition of 95.45% and 91.82%, respectively. The rest of the subinhibitory concentrations of the same sample induced the change in the biofilm-forming category to weakly

Table 2. MIC values of investigated essential oils against *C. albicans*

Tested compound	Essential oils				Nystatin
	C. limon	*C. reticulata*	*N. sativa*	*F. vulgare*	
MIC (µg/ml)	125	250	62.50	250	31.25

adherent, with inhibition of 73.75% to 85.79%. The highest antibiofilm activity in this study is recorded for *F. vulgare* EO, with 100% inhibition of the biofilm formation in the range of 125-31.25 µg/ml, while further dilutions also had great antibiofilm potential. The concentrations from 15.63 µg/ml to 3.90 µg/ml eliminated *C. albicans* biofilm (inhibition of 89.77-96.59%). The lowest tested dilution (1.95 µg/ml) caused the change in the biofilm-forming category to weakly adherent. The amount of biofilm inhibition by tested EOs is presented in Fig. 1.

Discussion

Considering the emerging resistance to synthetic drugs, as well as their potential toxicity, many investigations have been done to define novel compounds of plant origin with the ability to inhibit *C. albicans* (Gulati and Nobile, 2016; Shokri *et al*., 2016). However, studies regarding the antibiofilm activity of plant products against *C. albicans* are limited. This study tested the antifungal and antibiofilm properties of four essential oils derived from four edible and spice plants: *C. limon*, *C. reticulata*, *N. sativa,* and *F. vulgare*. All tested EOs in all established concentrations led to the formation of inhibition zones. The antifungal potential of the EOs is associated with hydrophobic molecules that attack ergosterol in fungal cell membranes, which leads to changes in membrane permeability and ultimately results in cell death. Molecules of EOs also interfere with the enzymes, which inhibits fungal growth (Prabajati *et al*., 2017).

The most prominent antifungal activity in the diffusion method was noted in the case of *N. sativa* EO, where inhibition zones were larger (28.30±1.50 - 39.30±1.10) in comparison to those induced by the standard antifungal drug Nystatin (21.30±0.60 mm). Unlike the precise chemical content of synthetic drugs, essential oils contain tens to hundreds of different compounds, and thus a single EO can exhibit different mechanisms of action against a microorganism. The resistance to the EOs is generally low since every constituent can be oriented to the specific cellular target (Tran *et al*., 2022). Most of the active compounds present in the EO of *N. sativa* are thymoquinone, *p*-cymene, carvacrol, *t*-anethole, 4-terpineol, and longifolene are able to interfere with the protein synthesis (Abdel-Latif *et al*., 2021). According to Azeiz *et al*. (2013), thymoquinone interrupts the function of the *C. albicans* cell membrane through the binding with nucleophilic amino acids. The study of Almshawit and Macreadie (2017) on *C. glabrata* noted that thymoquinone induces the generation of reactive oxygen species. The antifungal action of *N. sativa* could also be attributed to the presence of β-sitosterol, stigmasterol, and oleic acid (Abdel-Latif *et al*., 2021). The lowest MIC value for tested EOs is observed also in the case of *N. sativa* (62.50 µg/ml). The assessment of the antibiofilm properties of *N. sativa* EO against *C. albicans* suggests a strong activity, illustrated by elimination and high-percentage inhibition of biofilm detected in different subinhibitory concentrations. A previous study (Mustafa *et al*., 2018) discussed the antibiofilm features of *N. sativa* extract, but to the best of our knowledge, this paper represents the first description of the antibiofilm properties of the black cumin EO against *C. albicans*.

C. limon EO had MIC at 125 µg/ml, which is lower in comparison to the data published by Pedroso *et al*. (2019). The major compounds of *C. limon* EO are β-pinene, limonene, linalool, α-terpineol, linalyl acetate, geranyl acetate, nerolidol, neryl acetate, and farnesol (Ben Hsouna *et al*., 2017). Monocyclic and bicyclic monoterpenes (limonene, terpinene, and β-pinene) are associated with the antifungal properties of *C. limon* EO mainly through the inhibition of ergosterol synthesis (Hernawan *et al*., 2015). Radithia *et al*. (2022) noted that *C. limon* EO causes cyto-morphometric changes in *C. albicans*, as well as affects biofilm formation. These authors underline limonene as the compound responsible for the structural damage of ECM in *C. albicans* biofilm, which is in accordance with Pedroso *et al*. (2019). In our investigation, *C. limon* EO did not show prominent antibiofilm activity, at least in the context of changing the biofilm-forming category. Nevertheless, inhibition of the biofilm in the amount of 27.37% is noted at 7.81 µg/ml. The obtained result is still promising when compared to other studies that stated 2000 and 1000 µg/ml as the minimum biofilm formation inhibiting concentration and minimum biofilm-eradication concentration respectively (Pedroso *et al*., 2019).

The largest inhibition zones in the case of *C. reticulata* EO were achieved at 750 µg/ml, while MIC was determined at 250 µg/ml. According to Pedroso *et al*. (2019), MIC of the various *C. reticulata* EOs against *C. albicans* ranged from 1000 to >2000 µg/ml. Viuda-Martos *et al*. (2008) reported limonene and λ-terpinene as the main constituents of *C. reticulata* EO, accompanied by sabinene, linalyl acetate, copaene, and α-pinene (Boughendjioua *et al*., 2020). The antifungal activity of *C. reticulata* EO is previously reported (Denkova-Kostova *et al*.,

2020), but details regarding its antibiofilm activity are scarce. In our study, *C. reticulata* EO caused a decrease in the biofilm-forming category in the range of 125-7.81 µg/ml, with recorded inhibition of 50.68-70.68%. According to the available literature, this is the first study regarding the antibiofilm properties of *C. reticulata* EO against *C. albicans*.

F. vulgare EO had MIC at 250 µg/ml and exhibited great potential in terms of biofilm inhibition. All tested dilutions performed antibiofilm activity, including the total elimination of the *C. albicans* biofilm. *F. vulgare* EO contains many biologically active compounds, while the bicyclic monoterpene, fenchone, and anisaldehyde are suggested as responsible compounds for a large part of antimicrobial activity, including the antibiofilm properties against *C. albicans* (Ahmad *et al*., 2022). Monoterpenes, such as fenchone, interact easily with fungal cell walls and membranes, leading to changes in their permeability and therefore reducing the growth of biofilm (Manoharan *et al*., 2017). *In silico* analysis of the interaction between fenchone and 1,3-β-D-glucan synthase of *C. albicans* showed the formation of several H-bond interactions with particular amino acid residues and proposed this compound as a potential antibiofilm agent (Ahmad *et al*., 2022). Nevertheless, that specific research found that the MIC value of fenchone was 41.6±14.4 mg/ml, while in our study MIC of undiluted fennel EO was 250 µg/ml, which is significantly lower. That indicates that other constituents of tested EO could also be involved in antifungal activity.

This study indicated that the commercial antimycotic drug Nystatin exhibited a much lower MIC value in comparison to the tested EOs. Similar findings were presented in the investigation of Biernasiuk *et al*. (2023), who examined the activity of the clove EO and synthetic antifungal drugs against the same *C. albicans* strain. In that research, the MIC of Nystatin was recorded at 0.48 µg/ml, while EO had MIC at 1000 µg/ml. According to Nenoff *et al*. (2016) who studied *in vitro* susceptibility of yeasts to Nystatin, low MIC values suggested no indication of *in vitro* resistance of *C. albicans*, *Candida* species nor the non-*Candida* yeast species to this agent.

Although the biological activity of EOs is typically associated with the most abundant constituent, the study of Lawrence and Palombo (2009) suggests the involvement of the minor components, which can act synergistically with major compounds.

Conclusion

This study presented the essential oils of four edible and spice plants as antifungal and antibiofilm agents against *C. albicans*. Although this yeast is considered a regular member of the human microbiota, in particular cases, it could be involved in possible fatal infections, especially if it comes in the form of a biofilm. All tested plants: *C. limon*, *C. reticulata*, *N. sativa*, and *F. vulgare* exhibited antifungal and antibiofilm activity against the investigated strain of *C. albicans*. Available data regarding the previous studies suggest that this paper offers the first description of the antibiofilm properties of *C. reticulata* and *N. sativa* EOs against *C. albicans*. In general, EOs could be considered promising antifungal products of natural origin with possible use in many fields, from medicine and agriculture to food technology. Future studies should be oriented to the evaluation of particular bioactive compounds of EOs, their synergistic relationship with synthetic antimicrobial agents, investigation of the possible toxicity, and description of precise molecular mechanisms of antimicrobial activity.

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