https://doi.org/10.31925/farmacia.2025.2.19 **ORIGINAL ARTICLE**

SYNERGISTIC EFFECTS OF FLUCONAZOLE AND *ROSMARINUS OFFICINALIS* ESSENTIAL OIL AGAINST *CANDIDA* SPP. STRAINS

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Abstract

This study investigated the antifungal activity of *Rosmarinus officinalis* L. essential oil (EO) against selected *Candida* spp. strains with particular emphasis on its synergistic effect in combination with fluconazole. GC-MS analysis identified eucalyptol, camphor and borneol as the major constituents of the EO, all known for their antifungal properties. EO demonstrated significant inhibitory effects on fungal growth and adherence capacity to inert substratum, key factors in biofilm formation, virulence and pathogenicity. All tested strains exhibited resistance to fluconazole, however, the combination of rosemary EO and fluconazole showed strong synergistic effects against all *Candida auris* strains. These results indicate that *R. officinalis* EO enhances the antifungal efficiency of fluconazole. Stability assessment confirmed the chemical compatibility of the EO-fluconazole mixture under different storage conditions. The findings support the potential use of rosemary EO as an effective adjuvant in antifungal therapy, especially against multidrug-resistant *Candida* strains.

Rezumat

Studiul a investigat activitatea antifungică a uleiului esențial (UE) de rozmarin (*Rosmarinus officinalis* L.) asupra unor tulpini de *Candida* sp., precum si evidențierea efectului sinergic în combinație cu fluconazolul. Analiza GC-MS a arătat că principalele componente ale UE au fost eucaliptolul, camforul și borneolul, cunoscute pentru efectele antifungice. UE a demonstrat efecte inhibitorii semnificative asupra creșterii fungice și a capacității de aderența la substratul inert, factori esențiali în formarea de biofilme, virulență și patogenitate. Tulpinile testate au prezentat rezistență la fluconazol, în timp ce combinatia UE de rozmarin -fluconazol a demonstrat efecte sinergice, demonstrate prin valoarea FICI<0,5 în cazul tulpinilor de *Candida auris.* Aceste rezultate demonstrează că UE de rozmarin potențează eficiența fluconazolului. Testele de stabilitate au confirmat compatibilitatea chimică a amestecului UE-fluconazol în diferite condiții de păstrare. Rezultatele susțin utilizarea UE de rozmarin ca adjuvant în terapia antifungică, în special asupra tulpinilor multirezirestente de *Candida*.

Keywords: *Candida* spp., *R. officinalis* essential oil (EO), fluconazole, synergistic effect

Introduction

Nosocomial infections attributed to *Candida* species represent a considerable public health concern, exhibiting a rising prevalence and persistence within hospital settings, particularly in intensive care units. Among these, *Candida albicans* remains the most prevalent species, however, in recent years, non-*albicans* species such as *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and, in particular, *Candida auris* have emerged with increasing frequency and clinical significance due to their heightened resistance to antifungal agents and association with severe infections. *C. auris*, first identified in 2009, has rapidly become a multidrug-resistant pathogen with a high transmission potential and an aggressive epidemiologic profile, making it extremely difficult to manage [1, 2].

Fluconazole resistance represents a considerable challenge, especially in *C. auris*, which exhibits multidrug resistance and presents difficulties in eradication within hospital environments [3]. In addition, the biofilm formation capacity provides supplementary protection against antifungals and immune responses [4].

Considering the limitations of actual antifungal therapies and their frequent adverse effects, the development of

alternative treatment strategies is increasingly warranted. Essential oils (EOs), volatile natural compounds, have been intensively studied for their antifungal potential and ability to inhibit biofilm formation. Rosemary (*Rosmarinus officinalis* L.) EO has demonstrated significant antifungal effects against several *Candida* spp., including multidrug-resistant strains [5, 6]. It has been shown that among its main components, 1,8-cineole, camphor and α-pinene, contribute to the disruption of the fungal cell membrane and reduce the biofilm-forming capacity. Previous research confirms its antifungal and antibiofilm properties, making it a promising candidate for the development of alternative antifungal strategies [7].

This study aimed to evaluate the antifungal potential of *R. officinalis* EO against eight clinical *Candida* isolates associated with nosocomial infections, along with reference strains of *C. albicans, C. auris, C. parapsilosis*, and *C. tropicalis*. A key focus was to examine the effect of *R. officinalis* EO on fungal adherence to abiotic surfaces, a critical step in biofilm formation, which significantly contributes to pathogenicity and antifungal resistance. Furthermore, the research investigated the possible synergistic interaction between *R. officinalis* EO and fluconazole, to identify innovative therapeutic approaches to improve antifungal treatment outcomes.

Materials and Methods

R. officinalis EO characterization

The *R. officinalis* EO used in this study was purchased from a speciality store in the original packaging with a certificate of conformity and stored in sealed glass containers at 4°C until use.

Refractive index analysis

The refractive index analysis for EO was performed in the Physical Chemistry Laboratory of the Faculty of Pharmacy, UMF Carol Davila, Bucharest, using a Kruss DR 201-95 portable digital Refractometer for laboratory use. Sample droplets were placed with a pipette abundantly on the horizontal prism, then the prisms were blocked and the reading was performed according to the instruction manual [8].

GC-MS analysis

The essential oil (diluted 1:10 with hexane) was analysed by GC-MS using a Focus gas chromatograph coupled to a Polaris Q mass detector with electron impact ionisation (70 eV) and a Triplus autosampler (Thermo Fisher Scientific, Waltham, MA, USA). A DB-5MS capillary column (25 m length, 0.25 mm diameter and 0.25 μm film thickness) was used. The GC oven temperature was 60°C (3 min) increasing 10°C/min up to 200°C (2 min) and then 12°C/min to 240°C (2 min); the transfer line temperature was 250°C. The carrier gas was helium at a flow rate of 1 mL/min. Chromatogram analysis was performed using Xcalibur® 4.3 software, supported by the NIST 11 database for compound identification. The retention indices (RI) were calculated using the C8-C20 alkane standards (Sigma-Aldrich, Darmstadt, Germany) as a reference. Peak areas without correction factors were used to determine percent oil composition.

HPLC analysis

To evaluate the stability of the fluconazole-*R. officinalis* EO mixture, a high-performance chromatographic (HPLC) analysis was performed. Samples were monitored over a period of 30 days and stored under various environmental conditions: in the light and ambient temperature, in the dark and ambient temperature, in a refrigerator (4°C) and in a thermostat at 37°C. Freshly prepared samples (time T0), as well as separate controls of ROTICHROM® fluconazole (Merck, Germany) and *R. officinalis* EO were also analysed. The fluconazole calibration curve included values ranging from 46.65 to 746.4 μg/mL. Each sample was solubilised in methanol, followed by ultrasonication for 10 minutes. Chromatographic analyses were performed using an Agilent 1100 series HPLC system equipped with a Zorbax C18 column (100 mm x 3 mm, 3.5 μm). Detection was performed at 260 nm wavelength at a temperature of 22°C. The mobile phase was a mixture of acetonitrile (20%) and 0.1% phosphoric acid solution (80%) at a flow rate of 1 ml/min. The injection volume was 10 μl, and the retention time for fluconazole was 1.47 minutes [9, 10].

Stains identification

A total of eight strains of *Candida* spp. were isolated on Sabouraud Dextrose Agar (SDA) culture medium from different isolation sources from patients hospitalised at the National Institute of *Pneumophthisiology* "Marius Nasta" Bucharest, Romania, between January and May 2024. The identification was performed using the automated VITEK® 2 COMPACT system. In addition, four reference strains were investigated in the study: *Candida auris* DSM 21092, *Candida albicans* ATCC 10231, *Candida tropicalis* DSM 7524 and *Candida parapsilosis* ATCC 22019. All isolates were preserved in glycerol solution at -20 °C until use. The collection and use of nosocomial strains were performed with the approval of the Human Research Ethics Commission of the National Institute of *Pneumophthisiology* "Marius Nasta" (Approval no. 12255/17 June 2024), in accordance with the ethical provisions of the Declaration of Helsinki [11, 12].

Antifungal susceptibility profiles

The study assessed the antifungal susceptibility of *Candida* strains to fluconazole (FL, 0.016 - 256 μg/mL), micafungin (MYC, 0.002 - 32 μg/mL), amphotericin B (AMB, 0.002 - 32 μg/mL), caspofungin (CAS, 0.002 - 32 μg/mL) and flucytosine (FC, 0.002 - 32 μg/mL) using the E-test method to determine minimum inhibitory concentration (MIC) values. A standardised suspension of the tested strains, adjusted to a 1.0 McFarland standard in sterile water, was inoculated onto SDA plates. E-test strips were then

applied, and the plates were incubated at 37°C for 48 hours [13]. MIC values were analysed in accordance with the 2018 Clinical and Laboratory Standards Institute (CLSI) M60 guidelines.

Anticandidial activity of R. officinalis EO and fluconazole MIC determination

Quantitative evaluation of antifungal efficiency was performed using the serial binary microdilution method in 96-well plates. Dilutions were performed in the concentration range between 5 mg/mL and 0.009 mg/mL in RPMI 1640 culture medium (American Biorganics, Buffalo, NY, USA). A standardised suspension of yeasts, equivalent to 1 McFarland standard, obtained from the tested strains in sterile water to a final concentration of 10% in a total volume of 100 µL was added to each well. After incubating the plates for 24 h at 37°C, absorbance was measured at 620 nm using a Thermo Scientific™ Multiskan™ GO spectrophotometer. The MIC was defined as the lowest concentration of the test solution that completely inhibited fungal growth, determined from the values obtained in triplicate and adjusted by subtracting the blank values [14].

Influence of R. officinalis EO on microbial adherence to inert substratum

The effect of *R. officinalis* EO on the microbial adherence to an inert substratum was assessed using the crystal violet microtitration method. For this purpose, *Candida* strains were cultured in the presence of subinhibitory concentrations of the tested solutions (MIC/2 and MIC/4) following the protocol for quantitative antimicrobial assessment. After incubation, adherent cells were fixed with 99% methanol, stained with 1% crystal violet, and resuspended in 33% acetic acid. The percentage of adherence inhibition (PICA) was determined using the formula:

$$\%PICA = [(As-Ablank) * 100]/(Ac-Ablank),$$

where, As is the absorbance at 490 nm of treated samples and Ac is the absorbance at 490 nm of the control as previously described in [15].

Synergistic effects of fluconazole-R. officinalis EO combination against Candida spp.

The synergistic interaction between fluconazole and *R. officinalis* EO on *Candida* strains was investigated using the checkerboard microdilution method according to the CLSI protocol, with the necessary adaptations for this study. Briefly, fluconazole and *R. officinalis* EO were serially diluted in RPMI 1640 broth medium in 96-well microtiter plates, based on the MIC values previously determined for each tested strain. Each well was inoculated with a yeast suspension (a final concentration of 2.5 × 104 cells/well). Growth controls consisted of wells containing neither fluconazole nor essential oil. The range of concentrations tested for fluconazole ranged between 38.21 and 0.065 mg/mL, and serial dilutions corresponding to the effective concentrations determined above were used for *R. officinalis* EO. Plates were incubated at 37°C for 48 hours and the results were interpreted by calculating the fractional inhibitory concentration index (FICI) using the relationship:

FICI = (MIC of fluconazole in combination/MIC of fluconazole alone) + (MIC of rosemary essential oil in combination/MIC of rosemary essential oil alone).

The FICI values were interpreted as follows: FICI ≤ 0.5 indicated synergism; 0.5 < FICI ≤ 1 was considered an additive effect; 1 < FICI ≤ 2 irrelevant effect; and FICI > 2 antagonistic effect. Each determination was performed in triplicate for accuracy of results [4].

Statistical analysis

All experiments were conducted in triplicate to ensure reproducibility. Statistical analysis was performed using GraphPad Prism (version 10). To evaluate the impact of the combination of fluconazole with *R. officinalis* EO on the adherence capacity of *C. auris* strains to an inert substrate, a one-way analysis of variance (ANOVA) was applied. Subsequently, Dunnett's multiple comparison test was used to compare the treated groups with the untreated control groups.

Results and Discussion

Physico-chemical characterisation of R. officinalis EO

The chemical composition of the *R. officinalis* EO used in this study was determined by GC-MS analysis, and the compounds identified are included in Table I.

The *R. officinalis* EO analysed by GC-MS, identified 17 compounds that together make up approximately 99.83% of the total composition. The predominant components were oxygenated monoterpenes (87.12%), followed by non-oxygenated monoterpenes (9.52%). The main compounds identified were eucalyptol (52.83%), camphor (20.67%) and borneol (5.88%), which represent 79.38% of the total identified compounds. The chemical composition of EOs varies significantly depending on the extraction method, the period of plant harvesting, and the solvent used, according to the literature [16-20].

The presence of these compounds is particularly relevant because previous research has shown that α-pinenol and 1,8-cineole (eucalyptol) induce oxidative stress-dependent fungicidal action and inhibit the bio-film formation in *C. albicans* [21]. In addition, camphor and caryophyllene play an important role in destabilising the fungal cell membrane and disrupting mitochondrial functions. Moreover, the antifungal activity of mono-terpenes such as α-pinene and camphor has been confirmed against multiple *Candida* spp., including fluconazole-resistant strains, highlighting their role as potential therapeutic adjuvants [22]. According to Ivanov *et al*., camphor exhibits high anticandidal properties, including the ability to inhibit the biofilm formation and hyphal development in *Candida albicans*, while also reducing the expression of efflux pumps such

as CDR2, which are responsible for azole resistance [23].

The determination of the refractive index value (n) was carried out in triplicate and revealed an average value of n = 1.4650, in full correlation with those reported in the literature for high-purity rosemary EO [24].

Table I

R. officinalis EO composition determined by GC-MS

Compound	Retention indices (RI)	Relative content %
Non-oxygenated monoterpenes		
Acyclics		
myrcene	982	0.12
Cyclohexanes		
γ-terpinene	1049	1.03
α-terpinolene	1078	0.03
Bicyclics		
α-pinene	932	5.61
camphene	946	2.51
sabinene (*β*-thujene)	968	0.22
Total		**9.52**
Oxygenated monoterpenes		
Acyclics		
linalool	1087	1.12
Cyclohexanes		
eucalyptol	1024	52.83
Bicyclics		
camphor	1138	20.67
borneol	1158	5.88
Monocyclic alcohols		
α-terpineol	1180	5.33
Esters		
bornyl acetate	1274	1.29
Total		**87.12**
Phenylpropanoids		
eugenol	1344	0.03
Total		**0.03**
Non-oxygenated sesquiterpenes		
α-copaene	1363	0.01
β-caryophyllene	1409	3.06
Total		**3.07**
Oxygenated sesquiterpenes		
caryophyllene oxide	1582	0,03
Total		**0.03**
Other oxygenated non-terpenes		
benzyl benzoate	1704	0.06
Total		**0.06**
Total identify compounds		**99.83**

Given the complex composition and physicochemical characteristics of *R. officinalis* EO, it was deemed necessary to evaluate the stability of fluconazole in the presence of the EOs. For this purpose, an HPLC analysis was carried out, the results of which are presented in Table II. The choice of fluconazole was based on its frequent use as a first-line antifungal in medical practice in Romania. Since all *Candida* strains tested in the study showed resistance to this anti-fungal, the objective was to investigate the potential of potentiating the antifungal effect by association with *R. officinalis* EO. Prior to synergy testing, assessing the stability of this combination under various environmental conditions was an essential step to validate its therapeutic applicability.

HPLC analysis of the stability of fluconazole in combination with *R. officinalis* EO, carried out over a period of 30 days under controlled storage conditions, showed good chemical stability of the mixture. The percentage recovery ranged from 100.57% to 112%, with no signs of significant degradation, irrespective of exposure to light or temperature. The highest value was recorded for the sample kept in the dark at room temperature (112%), followed by the sample exposed to light (108.68%) and incubation at 37°C (105.30%). These minor variations can be attributed to storage

conditions but are within the accepted limits for pharmaceutical preparations [25]. Notably, the values exceeding 100% are most likely due to partial evaporation of the EO component, which led to a relative increase in the measured concentration of fluconazole. The findings agree with literature data showing high stability of fluconazole, even after exposure to varying temperature and light conditions for periods of up to 15 days [26].

Table II

HPLC analysis for fluconazole recovery in the presence of *R. officinalis* EO under different storage conditions after 30 days (T30)

Conditions	Time	Fluconazole (g)	Theoretical concentration (mg/mL)	Peak area	Measured concentration (mg/mL)	Recovery (%)
Dark, 4°C	T30	0,0355	0,3550	458,10	0,3570	100,57
Dark, 25°C		0,0315	0,3150	452,70	0,3528	112,00
Light, 25°C		0,0329	0,3290	458,80	0,3576	108,68
Dark, 37°C		0,0308	0,3080	416,20	0,3243	105,30
–	T0	0,0303	0,3030	420,30	0,3275	108,10

T0 = reference sample, analysed at preparation time

Distribution, identification and antifungal susceptibility of Candida strains

Eight clinical isolates belonging to four different *Candida* species, obtained from different biological sources were collected from patients hospitalised at the National Institute of *Pneumophthisiology* "Marius Nasta" in Bucharest, Romania, and subsequently analysed (Table III). Among them, four strains belonged to C. *auris*, recovered from urine (two stains), vaginal secretion and eschar samples. This reflects the ability of the *C. auris* species to colonise multiple anatomical sites, confirming its ecological versatility and increased pathogenic potential [27].

Two strains of *C. ciferrii*, isolated from perianal swabs and urine, as well as one isolate each of *C. glabrata* (from bronchial aspirate) and *C. lusitaniae* (also from bronchial aspirate), were also identified. We thus observe a diversified distribution, with a predominance of isolates from urine samples (three out of eight isolates), followed by respiratory secretions (two isolates from bronchial aspirates), which emphasises the tropism of these species for the urinary tract and airways, areas frequently affected in hospital settings.

Table III

Distribution of clinical *Candida* isolates by source of isolation, sex of patient and species identified

Strain code	Isolation source	Patient Sex	Identified Species
10	Bronchial aspirate	Male	*C. lusitaniae*
21	Perianal swab	Female	*C. ciferrii*
36	Bronchial aspirate	Female	*C. glabatra*
44	Vaginal discharge	Female	*C. auris*
68	Eschar	Female	*C. auris*
66	Urine	Male	*C. ciferrii*
99	Urine	Male	*C. auris*
19	Urine	Female	*C. auris*

The distribution of identified species in this study reflects the diversity and complexity of nosocomial fungal infections. *C. auris*, isolated predominantly from urine and skin secretions, confirms its emergent character and high potential for dissemination and antifungal resistance [28]. The presence of *C. glabrata* in the respiratory tract and *C. lusitaniae* in bronchial specimens emphasises the involvement of these opportunistic species in severe infections in immuno-compromised patients [29]. Also, identification of *C. ciferrii* supports its role in hospital colonisations, being associated with biofilm formation and resistance to conventional antifungal therapy [30].

Table IV

E-test results on antifungal susceptibility testing of *Candida* strains

Strain code		**10**	**44**	**19**	**36**	**96**	**66**	**68**	**21**
Antifungals - Interpretation according to the CLSI guidelines	**FL**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
	MYC	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
	FC	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
	CAS	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**
	AMB	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

R = resistant; S = sensible

Antifungal susceptibility results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, M60, 2018 edition, to determine the resistance or susceptibility profile to FL, MYC, FC, CAS and AMB of each tested strain. The findings are summarized in Table IV. According to the E-test results, all eight *Candida* isolates showed resistance to FL and AMB, while they were susceptible to MYC, CAS and FC [31].

It is important to note that official clinical breakpoints for *C. auris* have not yet been established by standard laboratory guidelines, which limits consistent interpretation of susceptibility data and necessitates reliance on provisional criteria and recent literature [32, 33]. In this context, the eight *C. auris* isolates in our study showed resistance to FL and AMB, but were sensitive to echinocandins and FC, a profile that reflects trends reported globally.

The adaptive genetic mechanisms described, such as mutations in the *PDR1* gene and overexpression of efflux pumps, are confirmed by *C. glabrata*'s reduced sensitivity to azoles [34]. Variability in the response of *C. lusitaniae* to amphotericin B is well-documented, associated with genetic changes in sterol biosynthesis pathways and a significant adaptive capacity [29]. *C. ciferrii* exhibited variable susceptibility, corroborating recent results that indicate a significant potential for biofilm formation and heightened resistance to azoles [35].

The lack of explicit breakpoints for these emerging species underscores the necessity for additional research and ongoing revisions of international guidelines to facilitate precise interpretation of laboratory data and optimal therapeutic strategies in nosocomial fungal infections.

Antimicrobial efficiency of R. officinalis EO

MIC determination for *R. officinalis* EO, in comparison with the control (DMSO), demonstrated a significant antifungal effect against all tested strains (Figure1). The MIC average values were lower for *R. officinalis* EO compared to the control, ranging from 0.102 µL/mL (in the case of *C. auris* DSM 21092) to 5 µL/mL (for *C. lusitaniae* encoded 10) ($p < 0.05$).

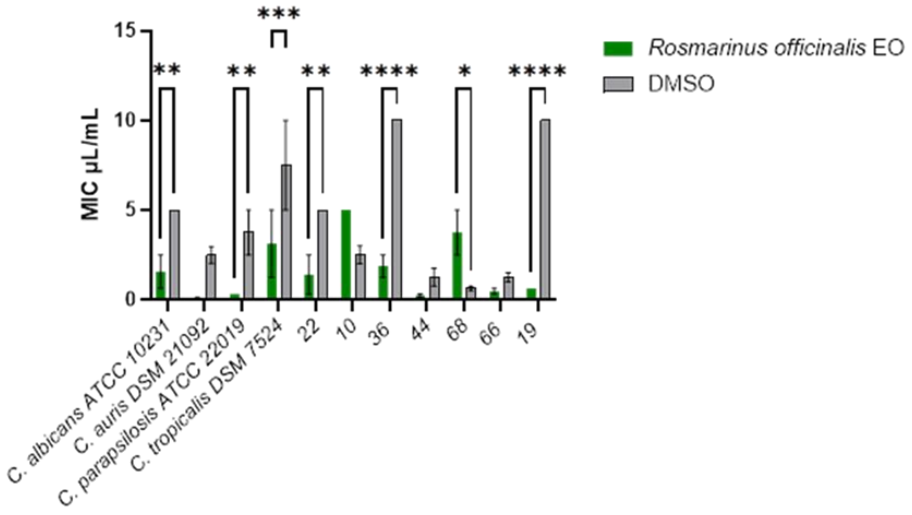

Figure 1.
Average MIC values for *Candida* spp. Strains
(* $p < 0.05$, ** $p < 0.001$, **** $p < 0.0001$) (Dunnett's multiple comparisons test)

MIC determination for *R. officinalis* EO demonstrated significant antifungal activity against *Candida* strains, with the lowest MIC values observed for *C. auris* (0.10 - 0.23 µL/mL), indicating a higher susceptibility. These findings are consistent with literature reports highlighting the efficiency of this EO against multi-drug-resistant *Candida* isolates [36]. Clinical isolates of *C. ciferrii* (encoded 21 and 66) exhibited MIC values of 1.40 and 0.46 µL/mL, respectively, indicating effective growth inhibition. These results are consistent with previous studies highlighting the antifungal potential of volatile compounds against emerging *Candida* species [37]. In the case of *C. glabrata* strain (encoded 36), the MIC value of 1.87 µL/mL indicates good anti-fungal activity that is significant in the light of the well-documented resistance of the organism towards conventional antifungals. In *C. lusitaniae* strain (encoded 10), the highest MIC value (5 µL/mL) was found, which may reflect the adaptive capacity of the species that relates to the differences reported in the literature depending on the chemistry of the oils and the cell wall composition [37].

The influence of *R. officinalis* EO on the adherence capacity of *Candida* strains to inert substratum was evaluated at sub-inhibitory concentrations (MIC/2 and MIC/4). The obtained results, shown in Figure 2, indicate a significant inhibition of adherence capacity (PICA%) at both tested concentrations compared to the control.

It was observed that adherence inhibition capacity was more pronounced at MIC/2 concentration, especially in the case of *C. glabrata* (encoded 36) and *C. parapsilosis*, where inhibition percentages were more than 60%. Notable inhibitory effects were also observed against *C. auris*, a species known for its persistence and resistance in hospital environments, highlighting the potential of *R. officinalis* EO in preventing biofilm formation.

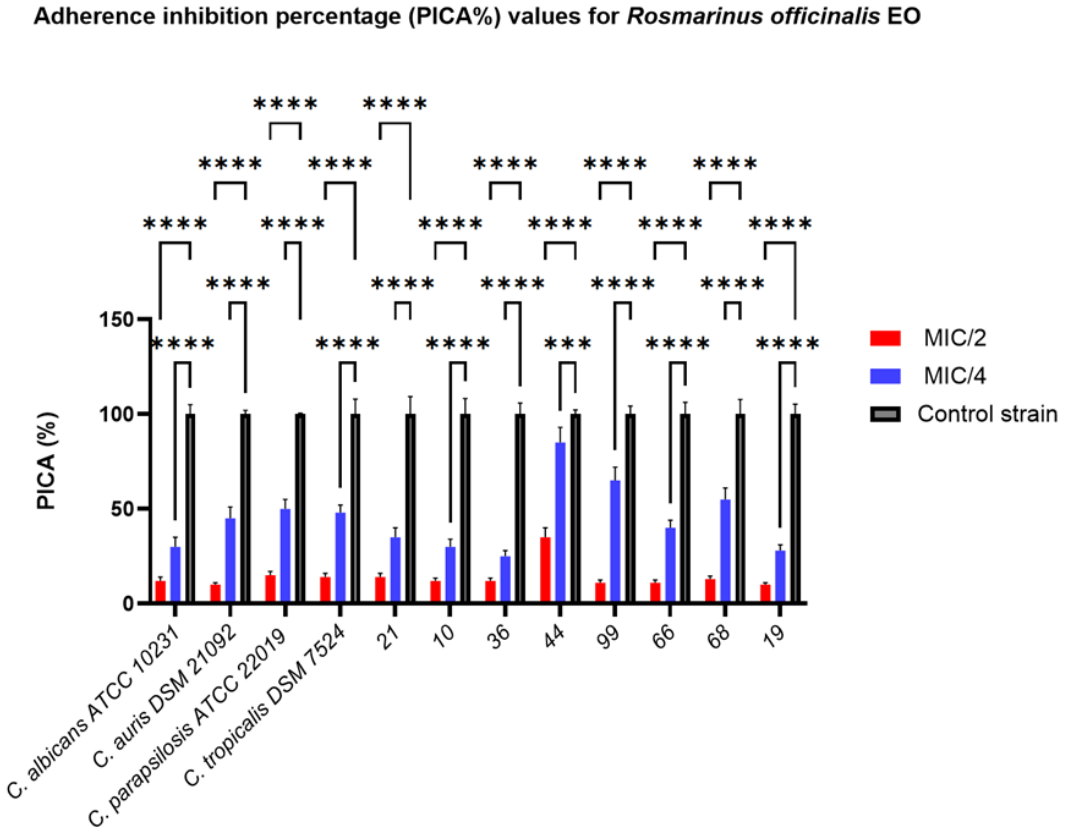

Figure 2.
Adherence inhibition percentage (PICA%) values for *R. officinalis* EO compared to the *Candida* strains (**** $p < 0.0001$) (Dunnett's multiple comparisons test)

Meccatti *et al*. emphasize the ability of bioactive compounds in *R. officinalis* EO, such as 1,8-cineole and camphor, to interfere with the adherence and biofilm formation in different *Candida* species [37].

Synergistic effects of fluconazole-R. officinalis EO combination

To assess the synergistic potential of the combination of fluconazole and *R. officinalis* EO, the microdilution method was applied and the results were interpreted by calculating the FICI values. The values obtained for each strain analysed are presented in Table V. They provide a detailed analysis demonstrating that the tested combination significantly reduces the MIC values of both active compounds, indicating a pronounced synergistic effect in the majority of cases.

Table V

Fractional Inhibitory Concentration Index (FICI) values for the combination of fluconazole and *R. officinalis* EO against *Candida* spp. Strains

Strains	MIC *R. officinialis* EO	MICa Combination	FICIa	MIC Fluconazole	MICb Combination	FICIb	FICI	Interpretation
C. albicans ATCC 10213	1,40	0,0500	0,0357	17	0,02	0,0012	0,0369	S
C. auris DSM 21092	0,14	0,0015	0,0107	17	0,03	0,0015	0,0122	S
C. tropicalis DSM 7524	1,40	0,0625	0,0446	17	0,02	0,0012	0,0458	S
C. prapsilosis ATCC 22019	0,31	0,0500	0,1613	32	0,02	0,0006	0,1619	S
C. auris 19	2,95	0,0900	0,0305	17	0,04	0,0024	0,0329	S
C. ciferrii 21	1,40	0,0313	0,0223	17	0,03	0,0018	0,0242	S
C. auris 99	1,40	0,0900	0,0643	38,21	0,04	0,0010	0,0653	S
C. auris 68	2,50	0,0900	0,0360	2	0,04	0,0200	0,0560	S

Strains	MIC *R. officinialis* EO	MICa Combination	FICIa	MIC Fluconazole	MICb Combination	FICIb	FICI	Interpretation
C. lusitaniae 10	2,50	0,0900	0,0360	32	0,04	0,0013	0,0373	S
C. glabrata 36	0,31	0,1250	0,4032	2	0,03	0,0125	0,4157	S
C. ciferrii 66	1,40	0,0152	0,0109	17	0,03	0,0018	0,0126	S
C. auris 44	1,40	0,0900	0,0643	17	0,04	0,0024	0,0666	S

MICa Combination = MIC of R. officinalis EO in combination; MICb Comnination = MIC of fluconazole in combination

The most pronounced synergistic effect was observed in case of reference strain of *C. auris* DSM 21092, where the FICI value was 0.0122. This extremely low value reflects a significant decrease in MIC for fluconazole (from 17 μg/mL to 0.03 μg/mL) and EO (from 0.14 μL/mL to 0.0015 μL/mL).

Also, in the case of clinical strains of *C. ciferrii* (encoded 66 and 21) was obtained very low FICI values (0.0126 and 0.0242, respectively), highlighting the sensitivity of these isolates to the combined treatment. It is important to point out that *C. ciferrii* is an emergent species with increased potential for biofilm formation and resistance to azoles [35].

The obtained FICI values for *C. albicans* ATCC 10231 and *C. tropicalis* DSM 7524 strains, recorded at 0.0369 and 0.0458 respectively, indicate robust synergism, corroborating prior experimental findings that highlight the antifungal efficacy of significant components in *R. officinalis* EO, including limonene, camphor and 1,8-cineole [5]. Also, these results confirm the findings of other research indicating the suppression of adhesion and biofilm formation in the presence of essential oils [37].

C. glabrata strain (encoded 36), although known to be intrinsically resistant to azoles, showed a synergistic response (FICI = 0.4157), less pronounced compared to the other strains. The decrease in MIC values from 0.31 μL/mL to 0.1250 μL/mL for oil and from 2 μg/mL to 0.03 μg/mL for fluconazole nevertheless confirms a significant therapeutic effect, despite the adaptive genetic mechanisms specific to this species [38].

The differences observed between the tested species in MIC and FICI values emphasize the importance of specific resistance mechanisms and cell structure. The superior response of *C. auris* strains to the combination treatment may be correlated with biofilm vulnerability to volatile compounds [3], while the lower efficiency observed in *C. glabrata* reflects its intrinsic mechanisms of azole resistance, such as overexpression of efflux pumps and mutations in regulatory genes [34]. Such variations support the need for species-differentiated therapeutic approaches.

The combination of *R. officinalis* EO with fluconazole diminished the MIC values for *C. parapsilosis*, *C. lusitaniae* (10), and *C. auris* (44) strains, with FICI values between 0.0373 and 0.0666. The results illustrate the universal character of the synergistic impact identified in this investigation, regardless of *Candida* species or clinical isolation source.

These findings confirm the ability of *R. officinalis* EO to potentiate the action of fluconazole and to overcome the barriers of antifungal resistance through complementary mechanisms such as destabilization of the cell membrane, disruption of mitochondrial functions and inhibition of biofilm formation [36].

The chemical composition of *R. officinalis* EO is essential for understanding its biological activity. The main compounds identified, such as 1,8-cineole (eucalyptol), α-pinene and camphor, have been shown to have significant antimicrobial and antifungal effects, either individually or synergistically [39, 40]. Eucalyptol can penetrate the fungal cell membrane and destabilise its integrity, thus facilitating the penetration of classical antifungal substances. About the potentiation mechanisms, it can be assumed that the essential oil enhances the efficacy of fluconazole through several pathways: increased membrane permeability and synergistic inhibition of ergosterol synthesis, the target mechanism of fluconazole [41]. In addition, interaction with the biofilm produced by *Candida* spp. is another important factor, as essential oils can prevent adherence and biofilm formation, thus reducing resistance to antifungal treatment [42].

Another important aspect is related to the phenomenon of synergism highlighted in recent studies on combinations of essential oils and azoles, including fluconazole. According to Parker *et al.* [3], EOs of lemon, cinnamon or clove demonstrated significant synergistic effects with fluconazole against *C. auris*, while combinations of compounds such as thymol and carvacrol potentiated the antifungal effect and reduced the MIC values [3, 43, 44]. In a comparable way, major compounds of *R. officinalis* EO may have the same beneficial effect. The combinations of essential oils with antifungal drugs are noted to reduce the resistance risk, as the pathogen is targeted through multiple and simultaneous mechanisms. Recent preclinical studies indicate that novel antifungal formulations utilising volatile compounds (such as cuminaldehyde, eugenol and carvacrol) may address the shortcomings of traditional antifungal therapies, particularly against multi-drug-resistant strains [45].

Our study emphasises that the combination of *R. officinalis* EO with fluconazole may be an effective strategy against multidrug resistant *Candida* strains. The observed synergistic effect suggests a relevant

therapeutic potential, especially in the context of reduced efficacy of azole monotherapy. However, the present study is limited by the absence of *in vivo* validations and an assessment of cell toxicity. The essential oils have low systemic bioavailability and high volatility, which may limit their systemic use, but recommend them in topical or mucosal formulations such as vaginal gels, oral solutions or skin hygiene products [46]. Also, the pharmacokinetics of the EO-fluconazole combination have not yet been explored, and possible interactions between volatile compounds and hepatic metabolism of fluconazole need to be investigated before large-scale clinical application. Thus, further preclinical studies, including toxicity assessments in cell culture and testing in animal models, are needed to validate the safety and efficacy of this therapeutic combination.

Conclusions

The obtained results demonstrate the significant therapeutic potential of *R. officinalis* EO in the control of fungal infections caused by multidrug resistant *Candida* strains. The intrinsic resistance, associated with inhibition of microbial adherence and biofilm formation, is complemented by a high synergistic effect in combination with fluconazole, confirmed by low FICI index values in all strains tested. *R. officinalis* EO is a promising candidate for the development of adjuvant or alternative antifungal therapies.

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Conflict of interest

Arsene AL is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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