UNIVERSITY OF MEDICINE AND PHARMACY
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STUDIES ON ANTIFUNGAL THERAPY RESISTANCE IN *CANDIDA* SPECIES
PHD THESIS ABSTRACT

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Contents

Introduction

Systemic fungal infections caused by species of the genus *Candida* are a major public health problem, which has increased in recent decades due to the increasing incidence and complexity of clinical manifestations, but especially due to the development of antifungal resistance. *Candida auris*, together with other *non-albicans Candida* species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. ciferrii*, are of increased clinical relevance due to the therapeutic and diagnostic difficulties they involve. These species are frequently associated with nosocomial infections and are characterized by varied resistance mechanisms, which significantly complicate the treatment and management of cases [1]. The World Health Organization has recently included *C. auris* in the list of priority, underlining the urgent need to develop therapeutic strategies innovative and effective [2].

Azoles are among the first-line antifungals widely used in clinical practice. Resistance to this class of drugs is becoming increasingly common, limiting current therapeutic options and requiring the identification of complementary treatment options [3].

In this study we set out to investigate both *in vitro* and *in vivo* the antifungal effect of combinations of fluconazole and a range of essential oils against a broad spectrum of *Candida* strains, including clinical strains isolated from hospital settings. The study makes novel contributions in modulating the inhibition of adhesion inhibition to the abiotic substrate, suppression of biofilm formation and modulation of fungal virulence factors. In parallel, the work evaluates the antifungal activity thiourea derivatives, structurally defined compounds that have shown inhibitory potential on fungal growth.

By exploring the therapeutic potential of natural and synthetic compounds, this work aims to make a significant contribution to the rationale for effective alternative therapies and to provide viable solutions for the control of resistant fungal infections, thus contributing to the improvement of clinical management and prognosis of patients.

1. GENUS *CANDIDA* - GENERAL CHARACTERIZATION AND CLINICAL IMPORTANCE

The genus *Candida* comprises a group of opportunistic fungi belonging taxonomically to the phylum *Ascomycota*, class *Saccharomycetes* and order *Saccharomycetales*. These unicellular yeasts are predominantly commensal micro-organisms inhabiting the oral cavity, gastrointestinal tract, urogenital mucosa and skin. The immune status of the host and environmental triggers determine their ability to change from a commensal state to a pathogenic form [4].

Over 200 species are currently attributed to the genus *Candida*, but only a limited number of these pose a threat to human health. *Candida albicans* is the most prevalent species responsible for fungal infectious pathologies globally [1]. The increasing clinical importance of *non-albicans Candida* species such as *C. glabrata, C. parapsilosis, C. tropicalis* and *C. auris*, characterized by increased drug resistance, has altered the epidemiological picture of candidiasis [5]. These species exhibit intrinsic resistance mechanisms and diverse pathogenic profiles, complicating diagnostic and therapeutic approaches.

2. THERAPEUTIC APPROACHES IN THE MANAGEMENT OF ANTIFUNGAL RESISTANCE IN *CANDIDA* SPECIES

Treatment of *Candida* infections is based on several major classes of antifungal agents, each with distinct mechanisms of action. The four main groups of antifungals currently in clinical use are azoles, echinocandins, polyenes and pyrimidine analogs. These agents remain the mainstay of antifungal therapy, although their efficacy is challenged by increasing resistance, particularly among *non-albicans C.* non-albicans species.

Azoles (e.g. fluconazole, voriconazole, isoconazole) inhibit the enzyme lanosterol 14-α-demethylase (encoded by the *ERG11* gene), disrupting the biosynthesis of ergosterol, an essential component of fungal cell membranes. Fluconazole is widely used for both prophylaxis and treatment of mucosal and invasive candidiasis, especially in resource-limited countries, due to its optimal benefit/risk ratio as well as cost. However, *C. glabrata* and *C. auris* frequently show resistance, which is associated with overexpression of efflux pumps and mutations in *ERG11* [6].

Empiric antifungal therapy can be initiated in patients at high risk of candidiasis, but targeted therapy should be followed once culture identification and antifungal susceptibility profiling are available [7]. Prophylactic strategies

are indicated in certain high-risk populations such as neutropenic cancer patients and premature newborns [8]. Special considerations apply to certain populations: neonates usually receive amphotericin B deoxycholate or fluconazole, and elderly patients and those with renal impairment require dose adjustment due to nephrotoxicities of the drug [9].

Antifungal resistance of *Candida* species is a growing challenge for current therapies, especially with the increasing prevalence of *non-albicans* strains of *C. non- albicans*, such as *C. glabrata* and *C. auris*. The mechanisms of resistance vary by antifungal class and are often multifactorial, involving genetic mutations, alterations in drug targets, overexpression of efflux pumps and biofilm formation.

Resistance to azoles is usually mediated by *single nucleotide polym*orphisms (SNPs) in the *ERG11* gene, which encodes lanosterol 14-α-demethylase, the therapeutic target of these drugs. These mutations reduce drug binding and confer cross-resistance to azoles. In addition, overexpression of ATP-dependent transporters increases drug efflux and reduces intracellular drug concentrations [10].

3. WORKING HYPOTHESIS AND GENERAL OBJECTIVES

In view of the worrying increase in resistance of Candida strains to conventional antifungals, especially azoles, this paper starts from the premise that the identification of alternative therapeutics is a priority in the management of fungal infections. In this context, the main hypothesis of the study is that certain essential oils and a series of synthetic thiourea derivatives may exert a relevant antifungal effect.

The study had the following general objectives:

1. Microbiological characterization of clinical strains of *Candida* sp.
2. Analysis of physico-chemical properties of selected essential oils.
3. Evaluation of *in vitro* antifungal activity of essential oils and thiourea derivatives.
4. Investigation of the synergistic effect between fluconazole and essential oils from *Rosmarinus officinalis* and *Cinnamomum verum.*
5. Evaluation of the efficacy of azole-phytocomplex combinations *in vivo* using the *Galleria mellonella* larval model.

6. Addressing alternative therapeutic perspectives based on natural and synthetic compounds that may complement or potentiate conventional antifungal therapy in the context of infections with resistant *Candida* strains.

4. GENERAL RESEARCH METHODOLOGY

The general research methodology was developed within the Department of Microbiology of the Research Institute of the University of Bucharest, with the approval of the Ethics Committee of the Institute of Pneumophthiology "Marius Nasta", according to the opinion no. 12255 dated June 17, 2024.

Materials used include reference strains of *Candida* sp., specific culture media (Sabouraud, CHROMagar Candida Plus, RPMI) and complex equipment (MALDI- TOF MS, UV/VIS spectrophotometer, VITEK® 2 Compact automated system, HPLC, etc.).

The methodology involves sampling, isolation and cultivation of clinical *Candida* strains from various pathological products (urine, eschar, vaginal discharge, bronchial aspirate), their microbiological characterization and the evaluation of the antifungal activity of thiourea derivatives and essential oils.

5. PHENOTYPIC CHARACTERIZATION OF THE ANTIFUNGAL RESISTANCE PROFILE OF NOSOCOMIAL *CANDIDA* STRAINS

This study aimed at the phenotypic characterization of 19 nosocomial *Candida* strains isolated from patients hospitalized at the Institute of Pneumophthiology "Marius Nasta" in Bucharest between January and May 2024. Also, possible correlations between antifungal susceptibility profiles, expression of virulence phenotypes and distribution of the isolated species were explored, in order to further characterize the local epidemiological picture.

The phenotypic characterization of nosocomial *Candida* isolates revealed a high prevalence of antifungal resistance, especially to fluconazole and amphotericin B. Despite the maintenance of susceptibility to echinocandins and flucytosine in the majority of isolates, the variability observed between strains emphasizes the need for species-level identification and individualized therapeutic approaches. Moreover, *in vitro* and *in vivo* analysis of virulence factors revealed important differences in enzyme expression profiles, suggesting that host-specific interactions play a key role in modulating pathogenic potential. The use of the *G. mellonella* model was effective in revealing

virulence factors that have not been observed standard culture conditions, emphasizing the value of integrating *in vivo* models in assessing *Candida* sp. pathogenicity. These findings contribute to a more comprehensive understanding of local epidemiology, antifungal resistance, and reinforce the need for continuous surveillance and improved diagnosis in the hospital setting.

6. STUDY OF THE ANTIFUNGAL AND ANTIOXIDANT ACTIVITY OF THIOUREA DERIVATIVES AGAINST NOSOCOMIAL STRAINS OF *CANDIDA AURIS* ISOLATED IN ROMANIA

Thiourea derivatives, known for their broad biological activities, may be promising therapeutic candidates. In the present study we aimed to characterize the antifungal activity on nosocomial strains of *C. auris*, isolated from clinics and the antioxidant activity of four such compounds synthesized in the Pharmaceutical Chemistry discipline of the Faculty of Pharmacy of the "Carol Davila" University of Medicine and Pharmacy of Bucharest [11].

Compounds tested:

In the Laboratory of Pharmaceutical Chemistry of the Faculty of Pharmacy of the University of Medicine and Pharmacy Carol Davila in Bucharest were synthesized from 2-thiophenacetic acid and thionyl chloride, four new organic compounds, which were characterized from the physicochemical point of view. Their structure was confirmed by ^{1}H-NMR and ^{13}C-NMR spectra by Bădiceanu, 2009 [11]. The structure of the compounds is depicted in Figure 6.1. The compounds appear as yellowish-white powders with increased stability, poorly hygroscopic, without being photosensitive or thermolabile. They are insoluble in water, soluble in organic solvents (dimethylsulfoxide - DMSO, alcohols), characteristic of nonpolar molecules [12].

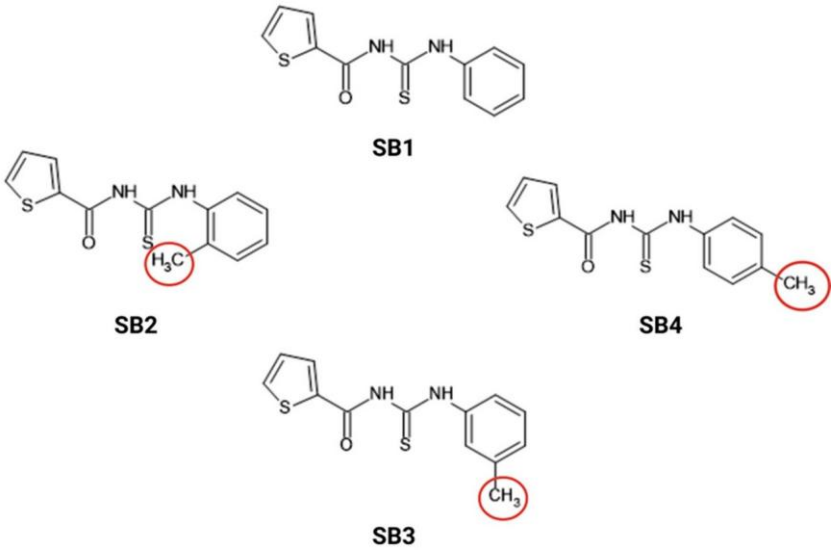

Figure 6.1. Chemical structure of thiourea derivatives

Testing of the antifungal activity of the four thiourea derivatives on *C. auris* strains by the adapted diffusimetric disc diffusion method revealed significant differences in antifungal activity. Among all the substances analyzed, SB2 showed superior activity, showing inhibitory effect on the growth of all tested strains. SB3 and SB4 showed a lower activity than SB2 each obtaining a UA1 score for only 11.11% of the strains. The weakest antifungal profile was observed for SB1, which did not inhibit the growth of any of the nine strains tested.

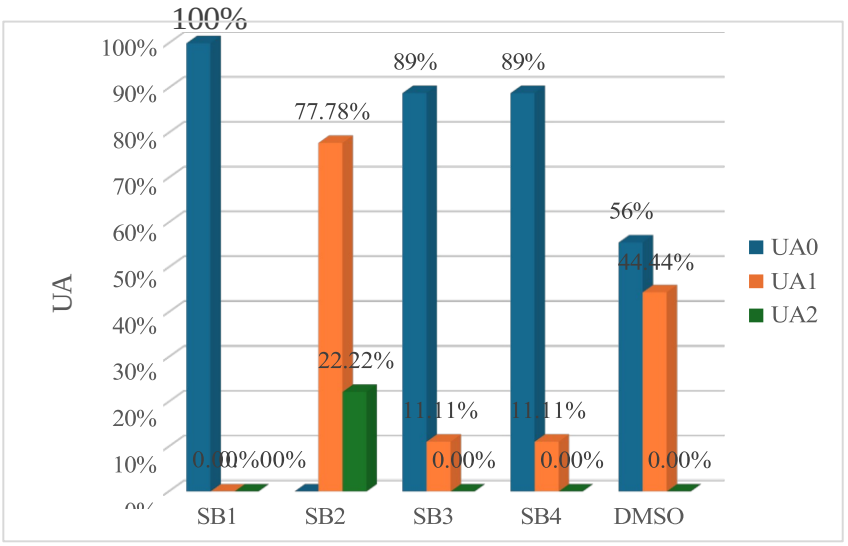

Figure 6.3. Distribution of arbitrary units determined by substances tested on *C. auris* strains

Concerning the quantitative evaluation of antifungal activity performed by microdilution method and determination of MIC value of thiourea derivatives on the analyzed *C. auris* strains, the results are included in Figure 6.4.

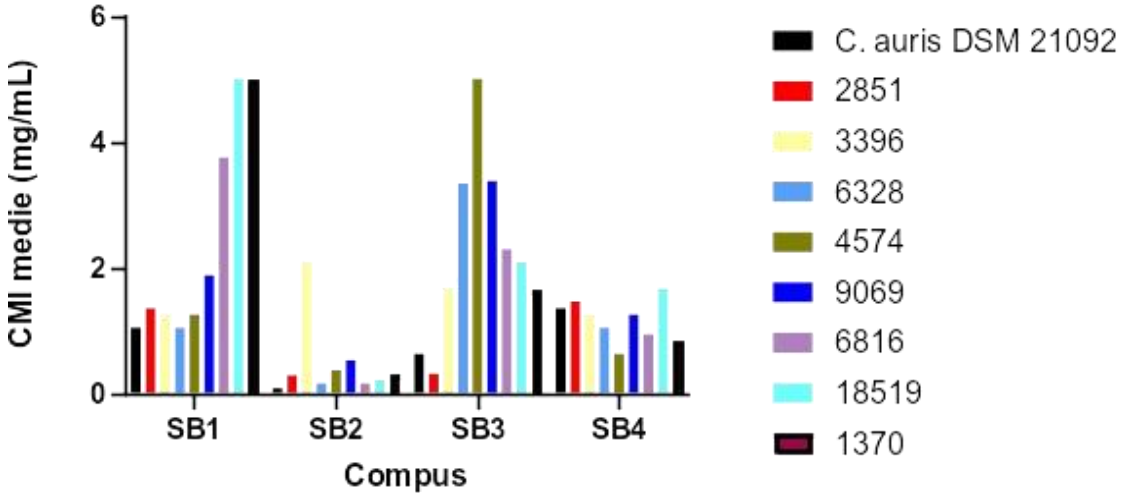

Figure 6.4. Representation of the mean values of the minimum inhibitory concentration values of the evaluated substances for the analyzed strains.

The position of the methyl group significantly affects antioxidant activity and hemolytic protection, with SB2 also demonstrating potent antioxidant and antihemolytic properties. These findings suggest promising therapeutic potential and warrant further clinical investigation.

7. COMPARATIVE STUDY OF THE ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS ON NOSOCOMIAL *CANDIDA* STRAINS

Essential oils (EOs), known for their complex mixtures of bioactive compounds, have shown promising effects in combating *Candida* infections due to their ability to inhibit fungal growth, disrupt biofilm formation and reduce the expression of virulence factors [13].

Among the most promising EOs are those extracted from aromatic herbs such as thyme (*Thymus vulgaris*), cinnamon *(Cinnamomum verum*), cloves (*Syzygium aromaticum*), rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*), which are rich in compounds such as thymol, carvacrol, eugenol and cinnamaldehyde. [13,14] Therefore, the investigation of the antifungal activity of these EOs against *Candida* clinical isolates may provide valuable information for the development of therapeutic strategies

natural and cost-effective alternatives for the management of fungal infections in the healthcare setting.

The results of this study confirm the antifungal potential of the selected EO, especially cinnamon, on nosocomial *Candida* strains isolated from a hospital in Bucharest. Of all those tested, cinnamon EO stood out with the lowest MIC values, suggesting a consistent and robust antifungal effect. This activity correlates with the high content of (E)-cinnamaldehyde, a compound known for its disruptive action on the fungal cell membrane and ability to induce oxidative stress.

Thymol and clove oils also showed notable antifungal activity, due to their increased content of thymol and eugenol, respectively, substances with an effect on cell membrane integrity and ergosterol synthesis. Oregano and rosemary variable efficacy, which could reflect differences in their chemical composition and in the way they interact with the cell walls of different *Candida* species.

An important aspect of the study was to evaluate the stability of fluconazole in combination with essential oils under various storage conditions. The results showed that the chemical structure of fluconazole is not adversely affected by the presence of the oils, which supports the idea of their use in combination formulations.

Beyond individual efficacy, this study opens interesting perspectives on the use of essential oils as adjuvants in conventional antifungal therapies. Combination with classical molecules, such as fluconazole, may enhance the therapeutic effect, reduce the required doses of antifungal and help to limit the development of resistance, a key objective in the context of nosocomial fungal infections, which are on the increase.

8. THE SYNERGISTIC EFFECTS OF FLUCONAZOLE AND *ROSMARINUS OFFICINALIS* ESSENTIAL OIL ON STRAINS OF *CANDIDA* SP.

This study aimed to evaluate the antifungal potential of *R. officinalis* EU against eight clinical isolates of *Candida* associated with nosocomial infections, together with reference strains *of C. albicans, C. auris, C. parapsilosis* and *C. tropicalis*. A key objective was to assess the effect of the EO of *R. officinalis* on fungal adhesion to abiotic surfaces, a critical step in biofilm formation, which contributes significantly to pathogenicity and antifungal resistance. In addition, the research investigated the possible synergistic interaction between *R. officinalis* EO and fluconazole, with the objective of identifying

potential innovative therapeutic approaches to improve antifungal treatment outcomes.

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

9. *IN VITRO* SYNERGISTIC EFFECTS OF FLUCONAZOLE AND *CINNAMOMUM VERUM* ESSENTIAL OIL ON *CANDIDA AURIS* STRAINS

The present study aims to evaluate *the in vitro* synergistic effects of fluconazole and *Cinnamomum verum* EO against nosocomial isolates of *C. auris*, providing further justification for the integration of phytotherapeutic compounds into antifungal treatment regimens.

Determination of the MIC for *Cinnamomum verum* EO, in comparison with the control (DMSO), showed a significant antifungal effect against all tested strains (Figure 8.1.). Mean MIC values were lower for *Cinnamomum verum* EO compared to the DMSO control.

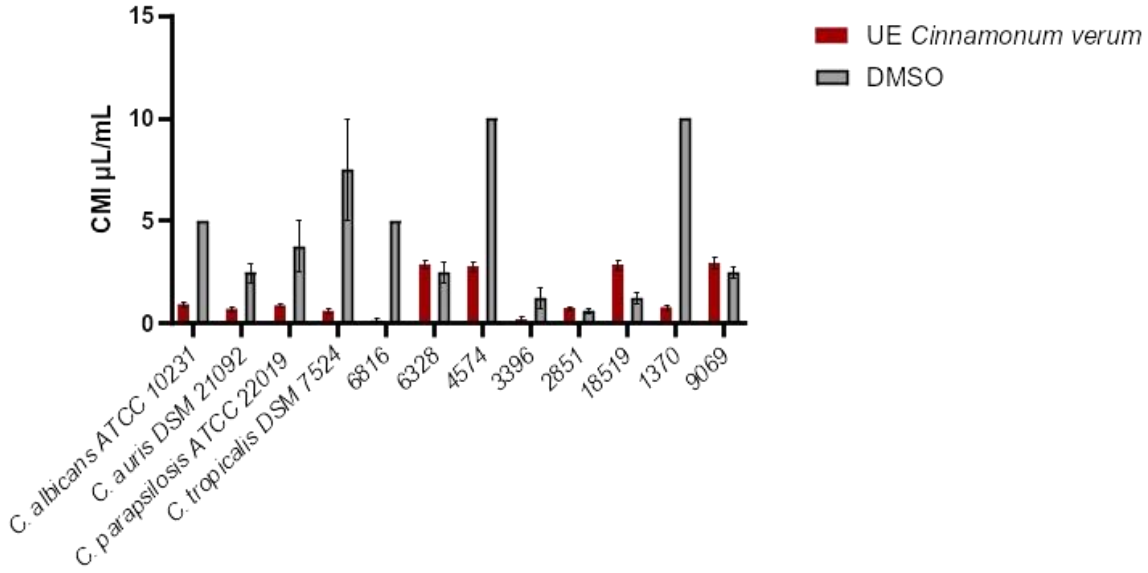

Figure 8.1. EO mean MIC values of *Cinnamomum verum* for all tested strains

The FICI index was calculated to evaluate *the in vitro* interaction between fluconazole and *Cinnamomum verum* EU against different *C. auris* strains.

The FICI results clearly demonstrate that the combination of *Cinnamomum verum* EO and fluconazole exhibits predominantly synergistic effects against different *Candida* strains, including MDR clinical isolates of *C. auris*.

The study demonstrates *the in vitro* synergistic antifungal activity of *Cinnamomum verum* EO and fluconazole against *C. auris* MDR strains. This antimicrobial effect is probably attributed to complementary mechanisms of action, cinnamaldehyde targeting fungal membrane integrity and fluconazole inhibiting ergosterol biosynthesis. In addition, cinnamon E) significantly inhibited fungal adherence to abiotic substrates of the tested strains, even at subinhibitory concentrations, suggesting an additional anti-virulence effect. These results support the integration of phytotherapeutic compounds, such as *Cinnamomum verum* EO, in antifungal treatment strategies, especially in combination with azoles, to enhance therapeutic efficacy and overcome the resistance phenomenon. Further *in vivo* studies and pharmacokinetic evaluations are needed to validate the therapeutic feasibility of these synergistic formulations.

10. IN VIVO EVALUATION OF THE SYNERGISTIC EFFECTS OF FLUCONAZOLE AND *CINNAMOMUM VERUM* ESSENTIAL OIL ON *CANDIDA* SP. STRAINS USING THE *GALLERIA MELLONELA* MODEL

In the present study, we aimed to evaluate *the in vivo* antifungal activity of fluconazole combined with *C. verum* EO against *Candida* sp. using the *G. mellonella* model, thus providing new insights into alternative antifungal strategies.

The *in vivo* study, using the *G. mellonella* model, demonstrated the superior antifungal effect of the combination of *Cinnamomum verum* EU and fluconazole compared to individual monotherapies, reflected by a significant reduction in larval mortality, fungal burdens and expression of key virulence factors such as hemolysins and lipases. These results support the inclusion of cinnamon essential oil as a therapeutic adjuvant in current antifungal regimens, highlighting its ability to potentiate the effect of fluconazole against *Candida* strains and providing a strong rationale for expanding preclinical research on synergistic natural-synthetic combinations.

CONCLUSIONS AND CONTRIBUTIONS PERONAL

The PhD thesis, by its aim and objectives, presents an innovative character due to its multidisciplinary approach (General and Pharmaceutical Microbiology, Analytical Chemistry, Physical Chemistry, Pharmaceutical Chemistry, Pharmacology, Phytochemistry) on new perspectives of treatment in *Candida* sp. infections, including multiresistant species.

In the current context, due to the emergence of the *C. auris* species, we wanted to identify new therapeutic strategies in the management of nosocomial infections due to this pathogen, which we have outlined in the first extensive study, so far, dedicated to *non-albicans Candida* species (*C. auris*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*) existing in the case casuistics of the Institute of Pneumophthiology "Marius Nasta" in Bucharest, Romania.

The research carried out in the PhD thesis aimed to identify alternative or complementary solutions for the treatment of infections with *Candida* species, in the context of the increasing number of strains resistant to available antifungal drugs. The formulated objectives were achieved by implementing various research methodologies, both *in vitro* and *in vivo*, and the results obtained allow the conclusions formulated below.

1. Phenotypic characterization of the antifungal resistance profile of nosocomial *Candida* strains.

This step allowed the selection of MDR strains for evaluation of their virulence and pathogenicity.

2. Study of antifungal and antioxidant activity of thiourea derivatives against nosocomial strains of *Candida auris* isolated in Romania.

We demonstrated for the first time, in this experimental context, that the positioning of the methyl group significantly influences the antioxidant activity and antihemolytic capacity of the tested compounds. SB2 was characterized by a complex bioactive profile, demonstrating antifungal, antibiofilm, antioxidant and antihemolytic properties, recommending it as a candidate molecule for future preclinical investigations.

3. Comparative study on the antifungal efficacy of essential oils on nosocomial strains of *Candida* sp.

We demonstrated medium-term (30 days) chemical compatibility of the mixtures, supporting the feasibility of using these combinations in stable antifungal formulations.

4. Synergistic effects of fluconazole and *Rosmarinus officinalis* essential oil on strains of *Candida* sp.

This study highlighted the ability of *Rosmarinus officinalis* essential oil to potentiate the antifungal activity of fluconazole, with potential translation to the clinic.

5. Synergistic effects of fluconazole and *Cinnamomum verum* essential oil on strains of *Candida* sp.

Cinnamomum oil (*Cinnamomum verum*) in combination with fluconazole showed *in vivo in* the experimental model on *Galleria mellonella*, a reduction in mortality of larvae exposed to treatments, reduction in fungal load and reduced expression of virulence factors, with complex impact on fungal pathogenicity.

The PhD thesis emphasizes the synergistic antifungal potential of conventional phytocomplex therapy combinations for translation to the clinic in an era where antifungal resistance is becoming a global public health problem.

As a future research direction, I intend to evaluate the biocompatibility of essential oil and fluconazole combinations to assess their potential for clinical application. In addition, I intend to pursue the development and optimization of a novel pharmaceutical formulation incorporating *Cinnamomum verum* essential oil and fluconazole, with the objective of increasing antifungal activity and reducing toxicity. This approach could provide a promising therapeutic alternative for the treatment of infections caused by multidrug-resistant strains of *Candida* sp.

Selected bibliography

[1] Guinea J. Global trends in the distribution of Candida species causing candidemia. Clinical Microbiology and Infection 2014;20:5-10. https://doi.org/10.1111/1469- 0691.12539.

[2] Ilie MI. Candida Auris: The Unwelcome Superfungus. Pharmacy 2023;71:225-35. Https://Doi.Org/10.31925/Farmacia.2023.2.1.

[3] Healey KR, Kordalewska M, Jiménez Ortigosa C, Singh A, Berrío I, Chowdhary A, et al. Limited *ERG11* Mutations Identified in Isolates of Candida auris Directly Contribute to Reduced Azole Susceptibility. Antimicrob Agents Chemother 2018;62. https://doi.org/10.1128/AAC.01427-18. https://doi.org/10.1128/AAC.01427-18.

[4] Farhan MS, Abdullah BA, Mamdwooh AE, Numan RS. Review of Virulence Factors in Candida. Journal for Research in Applied Sciences and Biotechnology 2024;3:75-82. https://doi.org/10.55544/jrasb.3.2.15.

[5] Pinho S, Miranda IM, Costa-de-Oliveira S. Global Epidemiology of Invasive Infections by Uncommon Candida Species: A Systematic Review. Journal of Fungi 2024;10:558. https://doi.org/10.3390/jof10080558. https://doi.org/10.3390/jof10080558.

[6] Chowdhary A, Prakash A, Sharma C, Kordalewska M, Kumar A, Sarma S, et al. A multicenter study of antifungal susceptibility patterns among 350 Candida auris isolates (2009-17) in India: role of the ERG11 and FKS1 genes in azole and echinocandin resistance. Journal of Antimicrobial Chemotherapy 2018;73:891-9. https://doi.org/10.1093/jac/dkx480. https://doi.org/10.1093/jac/dkx480.

[7] Tajane SB, Pawar S, Patil S. Revisiting the History of Candidiasis. Cureus 2025. https://doi.org/10.7759/cureus.78878. https://doi.org/10.7759/cureus.78878.

[8] Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Marr KA, Ostrosky-Zeichner L, et al. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. Clinical Infectious Diseases 2015;62:e1-50. https://doi.org/10.1093/cid/civ933. https://doi.org/10.1093/cid/civ933.

[9] Lass-Flörl C, Kanj SS, Govender NP, Thompson GR, Ostrosky- Zeichner L, Govrins MA. Invasive candidiasis. Nat Rev Dis Primers 2024;10:20. https://doi.org/10.1038/s41572- 024-00503-3.

[10] Bhattacharya S, Sae-Tia S, Fries BC. Candidiasis and mechanisms of antifungal resistance. Antibiotics 2020;9:1–19. https://doi.org/10.3390/antibiotics9060312.

[11] Daniela Bădiceanu C, Missir A-V. Synthesis And Characterization Of Some New Thioureides Of 2-Thiophenecarboxylic Acid With Potential Pharmacological Activity. Vol.
54. 2009.

[12] Carmellina Daniela Bădiceanu Dcna-Vmmhcdlmdmccl. Synthesis, Structural, Phisico-Chemical Characterization And Antimicrobial Activity Screening Of New Thiourea Derivatives. Farmacia Journal 2018;66:149-56.

[13] Palmeira-De-Oliveira A, Salgueiro L, Palmeira-De-Oliveira R, Martinez-De-Oliveira J, Pina-Vaz C, Queiroz Ja, Et Al. Anti-Candida Activity Of Essential Oils. Vol. 9. 2009.

[14] Potente G, Bonvicini F, Gentilomi Ga, Antognoni F. Anti-Candida Activity Of Essential Oils From Lamiaceae Plants From The Mediterranean Area And The Middle East. Antibiotics 2020;9:1-26. Https://Doi.Org/10.3390/Antibiotics9070395.