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THE *IN VITRO* ANTIFUNGAL AND SYNERGISTIC ACTIVITY OF SELECTED PLANT EXTRACTS AND ESSENTIAL OILS AGAINST *CANDIDA* SPP.

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ABSTRACT

This study aimed to evaluate the antifungal activity and the potential synergistic activity of ten plant extracts (EXs) and ten essential oils (EOs) obtained from the same plant species, namely *Agrimonia eupatoria* L., *Arctium lappa* L., *Arnica montana* L., *Bidens cernua* L., *Corylus avellana* L., *Echinacea purpurea* L. (Moench), *Menta piperita* L., *Trifolium pratense* L., *Syzygium aromaticum* L., and *Angelica sinensis* (Oliv.) Diels were used. Three species of the genus *Candida*, namely *C. albicans*, *C. glabrata*, and *C. tropicalis*, isolated from polluted estuarine water, were used in this study. The chemical composition of all EOs was evaluated by gas chromatography coupled with mass spectrometry (GC–MS) and gas chromatography with flame ionization detector (GC–FID) analysis. Firstly, the susceptibility of *Candida* spp. strains to EXs and EOs was screened by the agar disc diffusion method. Then the minimum inhibitory concentration (MIC) of the tested EXs and EOs was determined in a concentration range from 256 to 0.125 µL/mL. Following that, the effective EXs and EOs were divided into two groups (strong effect and weak effect) and tested in combination (strong + weak) at various concentrations depending on the tested strain to determine their potential synergistic effect using the chessboard pattern. The most sensitive strain to treatments with EXs and EOs tested alone and in mixtures was *C. albicans*. The most potent combinations with other EXs/EOs were observed with *Syzygium aromaticum* L., for both plant extract and essential oil, respectively. In this study the EOs and EXs tested in combination were much more effective than when acting on the growth of Candida spp. separately. So, the synergistic effect of the combinations of the EXs and EOs can lead to a reduction in the doses of commercial antifungals and can provide an effective way to reduce the infections caused by yeasts of the genus *Candida*.

Keywords: antifungal activity, *Candida*, essential oils, plant extracts, synergistic activity

INTRODUCTION

Yeasts of the genus *Candida* are commensals and also opportunistic pathogens capable of causing superficial and systemic infections **(Berman and Sudbery, 2002).** They cause a disease called candidiasis **(Thairu *et al*., 2019)**. Candidiasis is a general term that refers to skin, mucosal, and deep-seated organ infections that can occur at any age but primarily manifest in patients with weakened immunity **(Pappas *et al*., 2018)**. Recently, they have also gained importance as the causative agents of nosocomial infections **(Tsai *et al*., 2015)**. The prevalence of opportunistic infections caused by the genus *Candida* has increased, especially in immunocompromised individuals **(Douglas, 2003)**. Although most cases of candidiasis have been attributed to *C. albicans* species, in recent decades, improved diagnostic methods and higher levels of resistance to some fungicides have led to the discovery of NCAC (non-*C. albicans* Candida) species, especially *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* **(da Silva Lima *et al*., 2013)**. The pathogenicity of *Candida* species is facilitated by a number of virulence factors, including dimorphism, secretion of hydrolytic enzymes (proteases, lipases, and hemolysins), and the ability to form biofilms on medical devices or host mucosal epithelia **(Mayer *et al*., 2013)**. This variability increases the challenge of finding adequate therapeutic approaches that would be able to treat patients more effectively **(Cavalheiro and Teixeira, 2018)**.

Currently, therapeutic drugs for the treatment of *Candida* infections are limited to five classes of compounds: polyenes, allylamines, azoles, fluoropyrimidines, and echinocandins, which have different mechanisms of action **(Rabes *et al*., 2015)**. Resistance to these drugs is appearing due to the pervasive application of antifungal drugs such as fluconazole and voriconazole for prophylactic and therapeutic purposes **(Sultan and Ali, 2011)**. The fungistatic properties of some drugs, such as azoles and 5–flucytosine, also contribute to the development of resistance **(Wong *et al*., 2014)**, and biofilm formation can also contribute to and increase resistance **(Pierce *et al*., 2013)**. The lack of antifungal drugs and the simultaneous emergence of multidrug resistance (MDR) **(Fatima *et al*., 2020)** create an urgent need in the world to discover and find new synthetic or natural substances. The recent covid 19 pandemics has also shown that we are not ready to control the disease with available drugs. Authors **Sankar *et al*. (2021)** studied the potent phytocompounds from different medicinal plants (*Zingiber officinale* L., *Cuminum cyminum* L., *Piper nigrum* L., *Curcuma longa* L., and *Allium sativum* L.) by in silico analysis where also ADME/T (Absorption, Distribution, Metabolism, Elimination, and Toxicity) properties using the pkCSM server2 to predict their pharmacokinetic properties was used. The pre-diction showed that the identified phytocompounds (β-sitosterol, β-elemene and β-chlorogenin) present in the analyzed essential oils can be considered promising drug-like compounds that are additionally available. Compared to synthetic drugs, the natural products have many advantages, such as structural diversity and some of these have low toxicity **(Liu *et al*., 2017a)**. Natural products can supply a potential source of antifungal drugs **(Roemer *et al*., 2011)**, and given the utility of traditional medicine, it may be a promising strategy to develop antifungals from traditional natural medicines **(Liu *et al*., 2017b)**.

Substances derived from plants, such as hydroalcoholic extracts or plant essential oils, can play an essential role in this regard. Plant essential oils and extracts are usually complex mixtures of polar and non-polar natural compounds **(Macwan *et al*., 2016)**. They are known for their antiseptic and medicinal properties (analgesic, sedative, anti-inflammatory, anti-spasmodic, local anesthetic, and anticarcinogenic), and due to their antimicrobial and antioxidant activity, they are used as natural food additives and food products **(Liu *et al*., 2017a)**. The long-term and successful use of a combination of herbal medicines in traditional medicine has proven the synergistic effect of mechanisms based on multiple action targets **(Wagner *et al*., 2011)**. The possibility that one or more components of essential oil (EO) and plant extract (EX) interact synergistically with the components of another EO/EX has long been suggested as a drug with antimicrobial and antifungal effects **(Carson and Hammer, 2011)**. Synergistic combinations of

components such as eucalyptol in combination with thymol, p-cymene, or carvacrol against *Candida* spp. have been confirmed **(Pina-Vaz *et al*., 2004)**. Also, the synergy is not only influenced by the main components of EOs and EXs themselves because minor components can also have a decisive role, while the activity of the main components can be modified by these minor molecules **(Sahaf *et al*., 2008)**. There is also evidence of the synergistic effect of EOs and EXs with commercially available antifungals **(Pinto *et al*., 2009)**. For example, EOs damaging the cell wall and cytoplasmic membrane can synergistically facilitate their entry into the cell with fluconazole, which leads to a more significant effect on the inhibition of ergosterol biosynthesis. Such a synergistic combination can change the fungistatic effect of fluconazole into a fungicidal effect. In the case of amphotericin B, its toxicity is dependent on the dose used; in a syner-gistic combination with EOs or EXs, the toxicity will be reduced with the same effect. In addition, the potent fungicidal activity of the EOs and EXs against azole- and amphotericin B-resistant isolates implies that the EOs and EXs are effective against strategies or adaptive resistance mechanisms among *Candida* isolates against antifungal drugs **(Khan *et al*., 2012; Stević *et al*., 2014)**. This is because synthetic antifungals or antibiotics in the presence of EOs, thanks to their individual components (such as thymol, carvacrol, etc.) can enter the microbial cell faster and prevent the activation of pathogenic resistance **(Pirog, 2019)**.

So, there are many publications about EOs and EXs and their antibacterial and antifungal effects. Still, some have been tested against microorganisms only as essential oils or as extracts (methanol, aqueous, ethanol, acetone etc.) until now. Therefore, this study tested the antifungal properties of EXs and EOs from medicinal plants. Some of them have been shown to have antifungal properties, such as the water and ethanol extract from *Agrimonia eupactoria* L., which were effective against the yeast *C. albicans* and the strain *C. albicans* ATCC 10231 was the most sensitive to the acetone extract **(Muruzović *et al*., 2016)**, ethyl acetate extract from *Arctium lappa* L. was effective against *C. albicans* **(Petkova *et al*., 2022)** or chloroform extract of *Arnica montana* L. was tested against *C. albicans* **(Recio *et al*., 1989)**. Authors **Rybalchenko *et al.* (2010)** also proved that the phenylheptatriyne from *Bidens cernua* L. EO has significant antifungal activity against 70 clinical, 50 no-clinical and 5 standards reference strains of genus *Candida*. In the study of **Shataer *et al*. (2021)** were compounds obtained from *Corylus avellana* L. able to inhibit the proliferation of *Candida albicans*. The considerable growth inhibition on *Candida albicans* and *Saccharomyces cerevisiae* of *Echinacea purpurea* L. aqueous ethanol extract was shown in the study of **Stanisavljević *et al*. (2009)**. Also, antifungal activity of *Trifolium pratense* L. ethanolic extract has demonstrated good antifungal activity against *C. albicans*, *Aspergillus niger* and *Fusarium verticillioides* **(Arash, *et al*., 2013)**. In addition, many authors have already confirmed the antifungal activity of EXs or EOs obtained from *Metha piperita* L. **(Saharkhiz *et al*., 2012; Rajkowska *et al*., 2019)**, *Syzygium aromaticum* L. **(Pinto *et al*., 2009; Rajkowska *et al*., 2019)**, and *Angelica sinensis* (Oliv.) Diels **(Irshad *et al*., 2011; Ha *et al*., 2021)** against *Candida* species. But some of these plants (for example *Corylus avellana* L. or *Agrimonia eupactoria* L.), have never been tested as essential oil against fungi species yet. Furthermore, there is little or no information in the available literature testing their interactions leading to additive, synergistic or antagonistic effects.

Therefore, the main goal of this study was to evaluate the fungal growth inhibition of three species of the genus Candida (C. albicans, *C. glabrata*, and *C. tropicalis*) isolated from polluted estuarine water by selected ten EOs and EXs (alone and in mixture), both obtained from the same plant species mentioned above. Firstly, the antimycotic susceptibility of the tested isolates (*Candida* spp.) was tested. Next the antifungal effect of used EXs and EOs was tested by agar disks diffusion method and then the minimum inhibitory concentration (MIC) of the effective EOs and EXs were determined by using the microdilution method. Finally, the possible synergistic effect of effective EOs and EXs in mixtures was evaluated in different combinations by using the chessboard pattern.

MATERIAL AND METHODS

Essential oils (EOs) and plant extracts (EXs)

In this study, ten EXs and ten EOs obtained from the same plant species, namely *Agrimonia eupatoria* L., *Arctium lappa* L., *Arnica montana* L., *Bidens cernua* L., *Corylus avellana* L., *Echinacea purpurea* L. (Moench), *Menta piperita* L., *Trifolium pratense* L., *Syzygium aromaticum* L., and *Angelica sinensis* (Oliv.) Diels were used. The tested EXs were prepared by extraction from dried whole plant material. A 50 g of dried and crushed plant materials were soaked in 300 mL of ethanol p.a. (Sigma–Aldrich, Munich, Germany) for two weeks at room temperature in the dark. After two weeks, ethanolic plant extracts were filtered through the Watman No. 1 filter paper. Obtained plant extracts were subjected to evaporation under decreased pressure at 40 °C by a rotary evaporator (Stuart RE300DB, Bibby Scientific Limited, UK) with a vacuum pump (KNF N838.1.2KT.45.18, KNF, Munich, Germany). Ethanol evaporated, and crude plant extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Munich, Germany) to the final stock solution with a 102.4 mg/mL concentration. Final stock solutions of plant extracts were stored at -16 °C in the refrigerator until use. The tested EOs obtained by hydro-distillation (as stated by the manufacturer) were supplied by Calendula s.r.o. (Nová Ľubovňa, Slovakia) and Hanus (Nitra, Slovakia). The tested EXs and EOs were stored in closed, dark glass containers at 4±1 °C prior to analysis.

Chemical analysis of EOs

The chemical composition of all used EOs was analyzed using gas chromatography and mass spectrometry (GC–MS) (Agilent 7890A GC (HPST, s.r.o., Praha, Czech Republic) coupled to an Agilent MSD5975C MS detector (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS column (30 m × 0.25 mm, 0.25 µm-film-thickness) (HPST, s.r.o., Praha, Czech Republic)) and gas chromatography with flame ionization detector (GC–FID) (Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS, 30 m × 0.25 mm, 0.25 µm-film-thickness) techniques. The method, conditions used, and identification of the components were previously reported by **Hlebová et al. (2021a).** The components of the EOs were identified by comparing their mass spectra to relative retention indices (RI) against the National Institute of Standards and Technology Library (NIST, USA), as well as authentic analytical standards: (+)–α–Pinene, Camphene, (–)–β–Pinene, α–Phellandrene, p–Cymene, (R)–(+)–Limonene, Cineol,(–)–Linalool, Camphor, (+/–)–citronellal, (–)–Borneol, (–)–Menthol, 4–Terpineol, Estragole, Nerol, (–)–Carvone, Geraniol, Bornyl acetate, Thymol, Eugenol, β–Caryophyllene, α–Caryophyllene, Pentadecane, Farnesene, Myristicin, Caryophyllene oxide (Sigma-Aldrich, Munich, Germany) and available literature data **(Adams, 2007)**. The chemical composition of EOs was expressed in percent, and only components with a content above 1% were listed.

Fungal strains' origin and their identification

To test the inhibitory effect of EXs and EOs on the growth of *Candida* yeasts, the following species were used: *Candida albicans*, *Candida glabrata*, and *Candida tropicalis*. These strains were previously obtained from polluted estuarine water. The identification of *Candida* isolates was carried out using BD-Becton Dickinson *Candida* CHROMagar (Hi Media, Mumbai, India) and by MALDI-TOF MS (Bruker Daltonics, Munich, Germany, Maldi Biotyper) using single colonies of 48 h cultures of each strain according to **(Hleba *et al*., 2017)**. Spectra were analyzed using Flex Control 3.4 software (Bruker Daltonics, Inc., Billerica, MA, USA) and MALDI Biotyper OC version 3.1 (Bruker Daltonics, Bremen, Germany). After identification, all tested species were cultivated on Sabourad Dextrose Agar (SDA) (HiMedia, Mumbai, India) for 24-48 hours in the dark at 30±1 °C before inoculum preparation.

Preparation of *Candida* spp. inoculum before the EXs/EOs antifungal activity testing

Individual yeast colonies grown on SDA were suspended in 3 mL of sterile physiological solution and homogenized for 15 seconds using a vortex. Subsequently, the suspension was diluted with sterile physiological solution to a concentration of 2 to 5 x 10^6 CFU/mL. The obtained suspension was diluted in sterile distilled water (1:10) to a final inoculum of 2.5 x 10^5 CFU/mL, and the cell density was adjusted to the 0.5 McFarland turbidity standard. The inoculum of each tested *Candida* species was prepared 15–20 minutes before the analyses.

Screening of antifungal activity of EXs and EOs by agar disk diffusion method

The agar disk diffusion method was performed according to **Saracino *et al*. (2022)** with some modifications. Petri dishes (90 mm) containing SDA (HiMedia, Mumbai, India) were used to test the antifungal activity of 10 EXs and EOs. PDs were inoculated with 100 µL of prepared inoculum on the surface of the SDA medium. Then, 8 blank filter paper disks (Ø 6.0 mm) (Oxoid Thermofisher S.p.A, Milan, Italy) impregnated with 50 µL of each tested EX and EO were placed onto the agar surface. Firstly, the higher concentration of used EXs and EOs was used (512 µL/mL) to determine the sensitivity of tested *Candida* spp. strains to EXs and EOs. EOs were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Munich, Germany) (concentration of DMSO ≤ 3%) and 0.5% Tween 80 (Sigma–Aldrich, Munich, Germany). As negative controls, pure DMSO disks were used. Plates were incubated at 37±1 °C for 48 h. After cultivation, inhibition zones were measured with a digital caliper in two mutually perpendicular planes. The created zones of tested *Candida* spp. strains under EXs/EOs treatments were evaluated as follows:

- ≥ 12 mm (twice the size of the disk), the tested EXs/EOs had an inhibitory effect (IE) on yeast growth;
- ≤ 12 mm, the tested EXs/EOs had an average effect (AE) on yeast growth;
- < 12 mm or 0 mm, the tested EXs/EOs had no effect (NE) on yeast growth.

Determination of the minimum inhibitory concentration (MIC) by the microdilution method

The minimum inhibitory concentration (MIC) was tested by the microdilution method, according to **Khan *et al*. (2017)**, with a minor modification. The test was performed on 96–well microtiter plates. EXs and EOs (only those EXs and EOs which inhibited the growth of tested strains at a concentration of 512 µL/mL were selected) were tested in the concentration range from 256 to 0.125 µL/mL. The EOs were diluted in the same way as described above (chapter 3.6). 100 µL of Sabouraud broth medium (SBM) (HiMedia, Mumbai, India) was added to each

well. To the 1st column, a 100 µL of EXs/EOs (1st EXs/EOs = wells A2 to C2; 2nd EXs/EOs = wells E2 to G2) at a concentration of 256 µL/mL in 3 repetitions were added. The individual EXs and EOs were separated from each other by the control lines (D1 to D6: positive control of *Candida* spp. growth; D7 to D12: control of the medium purity; H1 to H6: purity control of the 1st EXs/EOs; H7 to H12: purity control of the 2nd EXs/EOs), which served as a control. 100 µL of each EO or EX was transferred using the two-fold dilution method from row 1 to row 12. Subsequently, 100 µL of the prepared inoculum was applied to all the wells (except lines D7 to D12 – control of the medium purity). The final volume in each well was 200 µL. Next, the microtiter plates were measured at 630 nm in the Opsys MRTM Microplate Reader to obtain the initial data. The prepared microtiter plates were cultivated for 48 h at 37±1 °C. After incubation, the microtiter plates were measured again and processed to determine the MIC for each tested EX or EO.

Determination of the synergistic effect of tested EXs/EOs

Based on the MIC determination results, the EXs/EOs with a weak inhibitory effect (EXs with MIC values ranging from 128 to 64 µL/mL; EOs with MIC values ranging from 64 to 16 µL/mL) and those with a strong inhibitory effect (EXs with MIC values ranging from 32 to 0.125 µL/mL; EOs with MIC values ranging from 8 to 0.125 µL/mL) were chosen to assess their potential synergistic effect in combinations (EXs/EOs with a weak effect + EXs/EOs with a strong effect). EXs/EOs with a very poor inhibitory effect (EXs with MIC values ranging from 512 to 256 µL/mL; EOs with MIC values ranging from 512 to 128 µL/mL) were excluded from this experiment. Thus, the EXs/EOs were tested from their lowest concentration obtained in the MIC evaluation, and the final tested concentrations of EXs/EOs. The inhibitory effect of individual EXs/EOs also depended on the fungal species tested, so for each tested strain, a different concentration was used (Table 1). Evaluation of the synergistic effect of the double combination of EXs and EOs was performed on the 96-well microtiter plate using the checkerboard method adapted from the methodology of **Hlebová *et al.* (2021b).** The final concentration tested was prepared separately for each EX/EO in microtubes. Each well received 100 µL of SBM. Next, the individual concentrations of the tested EXs and EOs were applied to a 96-well microtiter plate from the 1st column to the 9th column (lines A–D), and a two-fold dilution method was used to form the mixture of EXs and EOs. The EXs and EOs were added in the following order (to make a combination weakEXs/EOs + strongEXs/EOs): the weak EX/EO was added into lines A–H and strong EX/EO into columns 1–9. Column 10 (lines from A to D for the first EX/EO and lines E to H for the second EX/EO) served as a purity control for the tested individual EXs and EOs. Only pure medium SBM was used in column 11 (control of medium purity), and SBM with an inoculum of the tested strain was used in column 12 (positive control of *Candida* spp. growth). The final volume of each well was 200 µL. After inoculation, the plates were measured, cultivated, and evaluated in the same way as described in the method used to determine the minimum inhibitory concentration (MIC).

Table 1 The final concentration (µL/mL) of EXs (with weak or strong inhibition activity) and EOs (with weak or strong inhibition activity) against each different species of the genus *Candida* used for synergistic effect evaluation

EOs with weak inhibitory effect	**Range of tested concentration (µL/mL)**	**Tested fungi**
Corylus avellana L.	64 - 0.5	*Candida albicans* *Candida glabrata*
Echinacea purpurea L. (Moench)		*Candida albicans*
Trifolium pratense L.		*Candida tropicalis*
EOs with strong inhibitory effect	**Range of tested concentration (µL/mL)**	**Tested fungi**
Syzygium aromaticum L.	0.25 - 0.0009 4 - 0.0156 2 - 0.0078	*Candida albicans* *Candida glabrata* *Candida tropicalis*
Angelica sinensis (Oliv.) Diels	2 - 0.0078 8 - 0.0312	*Candida albicans* *Candida glabrata* *Candida tropicalis*
Bidens cernua L.	4 - 0.0156 32 - 0.1250 16 - 0.0625	*Candida albicans* *Candida glabrata* *Candida tropicalis*
Menta piperita L.	0.5 - 0.0019 8 - 0.0312 4 - 0.0156	*Candida albicans* *Candida glabrata* *Candida tropicalis*
EXs with strong inhibitory effect	**Range of tested concentration (µL/mL)**	**Tested fungi**
Syzygium aromaticum L.	4 - 0.0156 32 - 0.1250 16 - 0.0625	*Candida albicans* *Candida glabrata* *Candida tropicalis*
Angelica sinensis (Oliv.) Diels	16 - 0.0625 32 - 0.0312	*Candida albicans* *Candida tropicalis*
Bidens cernua L.	8 - 0.0312	*Candida albicans*
Menta piperita L.	16 - 0.0625 32 - 0.1250	*Candida albicans* *Candida tropicalis*
EOs with weak inhibitory effect	**Range of tested concentration (µL/mL)**	**Tested fungi**
Trifolium pratense L.	128 - 1	*Candida albicans*
Echinacea purpurea L. (Moench)		*Candida albicans*
Angelica sinensis (Oliv.) Diels		*Candida glabrata*
Menta piperita L.		
Bidens cernua L.	64 - 0.5 128 - 1	*Candida glabrata* *Candida tropicalis*
Corylus avellana L.	128 - 1	

Result evaluation

To express the antifungal effect of the combination of two tested EXs/EOs, the fractional inhibitory concentration index (FICI) was calculated according to:

FICI = (FIC1/MIC1) + (FIC2/MIC2)

where FIC1 and FIC2 represent the fractional inhibitory concentrations of combined EOs and EXs, and MIC1 and MIC2 represent the minimum inhibitory concentrations of individual tested EXs and EOs. Based on the obtained FICI values, the effect of the EXs/EOs combination was evaluated as follows: synergistic (FICI ≤ 0.5), partial synergistic effect (0.5 < FICI ≤ 0.75), no effect (0.75 < FICI ≤ 1.5), and antagonistic (FICI ≥ 2) **(Hlebová *et al.*, 2021b)**.

Statistical evaluation

All experiments were performed in triplicate in this study. The Microsoft Office Excel computer software and the Statgraphics Centurion XVI program (version 16.1.11) were used for statistical analysis of the results (one-way ANOVA and Tukey HSD 95% multiple range test; $p<0.05$). The results of MIC_{50} (MIC at which 50% of microorganisms are inhibited) and MIC_{90} (MIC at which 90% of microorganisms are inhibited) were evaluated using probit analysis in Statgraphics Centurion XVI program (version 16.1.11).

RESULTS AND DISCUSSION

Sensitivity of *Candida* spp. strains to EXs/EOs tested by agar disks diffusion method

To determine the sensitivity of *Candida* spp. strains to EXs and EOs, the highest concentration (512 µL/mL) was tested first. EXs and EOs were considered effective if the inhibition zone formed around the growth of particular fungi was equal to or twice (minimum 12 mm) the size of the disk that was being used (6 mm). None of the tested *Candida* spp. strains showed sensitivity to the EXs/EOs of *Arnica montana* L. and *Agrimonia eupactoria* L. (Table 2). Similar to our

research, **Koo *et al*. (2000**) tested the plant extract from *Arnica montana* L. and found that it had no inhibitory effect on the growth of *Candida albicans*. Many authors have reported that *Arnica montana* L. extracts (ethanol, methanol, etc.) are effective against bacteria such as *Helicobater pylori* **(Castillo-Juárez *et al*., 2009)**, *Streptococcus mutans* and *Phorphyromonas gingivalis* **(Rosas-Piñón *et al*., 2012)**, and fungi such as *Microsporum gypseum* CECT 2908 and *Trichophyton mentagrophytes* CECT 2795 **(Freixa *et al*., 1998)** but their effectiveness is clearly lower compared to available antifungals and antimicrobial agents such as metronidazole, amoxicillin or nystatin and amphotericin.

Table 2 Means of inhibition zones diameter in mm ± SD for individual *Candida* strains tested (3 repetitions were used for each strain (n=3)) treated with individual EXs and EOs

Plant species	Tested fungi					
	Candida albicans		*Candida glabrata*		*Candida tropicalis*	
	EXs	EOs	EXs	EOs	EXs	EOs
	Means of inhibition zones diameter (mm) ± SD					
Arnica montana L.	NE	NE	NE	NE	NE	NE
Arctium lappa L.	12.00^{a}±1.00	17.00^{a}±0.79	-	-	-	-
Agrimonia eupatoria L.	NE	NE	NE	NE	NE	NE
Bidens cernua L.	17.10^{bc}±0.01	22.00^{b}±1.00	18.50^{b}±0.46	20.00^{c}±2.00	14.50^{c}±0.44	16.10^{c}±0.40
Corylus avellana L.	16.80^{b}±0.01	18.20^{a}±0.35	-	16.40^{b}±0.40	13.40^{b}±0.30	16.70^{d}±0.20
Echinacea purpurea L. (Moench)	18.30^{c}±0.01	23.10^{b}±0.10	-	13.20^{a}±0.56	12.00^{a}±1.00	14.10^{b}±0.10
Menta piperita L.	22.00^{d}±1.00	25.60^{e}±0.79	14.60^{a}±0.30	16.80^{b}±0.26	21.10^{e}±0.10	24.06^{e}±0.05
Trifolium pratense L.	17.90^{bc}±0.01	22.50^{b}±0.20	-	-	12.30^{a}±017	13.40^{a}±0.20
Syzygium aromaticum L.	32.40^{e}±0.01	NG	25.50^{c}±0.56	NG	20.00^{d}±1.00	NG
Angelica sinensis (Oliv.) Diels	23.00^{d}±0.01	25.60^{e}±0.79	17.80^{b}±0.36	21.15^{c}±1.48	22.30^{f}±0.20	26.20^{f}±0.10

Legend: - – no inhibition zone was formed, NG – no growth was observed, the growth was completely inhibited by EXs/EOs, NE – no effect, SD – standard deviation; Data in the column followed by different letters are significantly different in 95.0% Tukey HSD test, $p < 0.05$.

Candida albicans was the most sensitive strain tested for EXs and EOs. According to our results, both the plant extract (12.00 mm) and essential oil (17.00 mm) from *Arctium lappa* L. inhibited the growth of *C. albicans*. The growth of *C. glabrata* and *C. tropicalis* was not affected by this EX/EO. Authors **de Oliveira Pereira *et al*. (2015)** confirmed the inhibitory activity of this extract against *Candida albicans* as well as *C. glabrata* using the microdilution method, but their tested concentration was higher (MIC_{90} 125 mg/mL and MIC_{50} 62.5 mg/mL). Good inhibition activity was shown with EXs/EOs from *Trifolium pratense* L. against two tested strains: *C. albicans* (EX 17.90 mm; EO 22.50 mm) and *C. tropicalis* (EX 12.30 mm; EO 13.40 mm). *C. glabrata* was the most resistant fungus to treatment with EXs/EOs in our study. However, the authors **Budzyńska *et al*. (2014)** tested 85% methanol extracts of *Trifolium alexandrinum*, *T. incarnatum*, and *T. resupinatum* against *C. albicans* and *C. glabrata* (resistant to fluconazole). The MIC of these extracts able to inhibit the growth of *Candida albicans* was determined at a concentration of 0.25 µg/mL and the growth of *C. glabrata* was also inhibited with a higher MIC value of 64 µg/mL, similarly to our study. In this study, *Bidens cernua* L., *Menta piperita* L., and *Angelica sinensis* (Oliv.) Diels were considered very effective EXs/EOs against all tested strains of the genus *Candida*. Many authors report the effectiveness of these extracts and essential oils against different strains of the genus *Candida* **(Rybalchenko *et al*., 2010; Tomczykowa *et al*., 2017; Norouzi *et al*., 2021)**, fungi **(Beck and Stermitz, 1995; Tabanca *et al*., 2008)**; and bacteria **(Han and Guo, 2012)**. In our study, the most effective EX and EO were from *Syzygium aromaticum* L.. The means of inhibition zones diameter under treatment with this extract (32.40 mm for *C. albicans*; 25.50 mm for *C. glabrata*; and 20.00 mm for *C. tropicalis*) were almost double compared to other tested extracts. **Khan and Ahmad (2012)** used this extract together with the antifungal amphotericin B, and the results suggest that clove significantly increased the effectiveness of amphotericin B against resistant strains of *Candida albicans* and may be very important in the future in the treatment of candidiasis. In our study, the most resistant to treatment with *Syzygium aromaticum* L. extract was *C. tropicalis* (mean of IZ diameter was 20.00 mm). Our results agree with those of authors **Suman *et al*. (2017)**, who tested this extract against *C. tropicalis* and *C. glabrata*. After 48 hours, the inhibition zones measured 36 mm for *C. glabrata* and only 20 mm for *C. tropicalis*. In the case of clove essential oil, the sizes of the inhibition zones were not determined because it caused complete inhibition of the growth of all tested *Candida* strains and no measurable zones were formed. Interestingly, **Alshaikh and Perveen (2017)** also tested the inhibitory effect of clove essential oil by the disc diffusion method at a concentration from 100 to 2% (v/v) on the growth of 20 clinical isolates of *C. albicans*. In their study, the size of the inhibition zones varied from 8 mm to 33.7 mm.

Minimum inhibitory concentration (MIC) determination

Based on the results of antifungal activity testing by the disc diffusion method, EXs/EOs that had no inhibitory effect on the tested species of the genus *Candida* were excluded from the next experiment (*Agrimonia eupactoria* L. and *Arnica montana* L. for all tested strains; *Arctium lappa* L. for *C. glabrata* and *C. tropicalis*). The results are summarized in Table 3.

Four EOs, namely *Bidens cernua* L., *Menta piperita* L., *Syzygium aromaticum* L., and *Angelica sinensis* (Oliv.) Diels showed a high inhibitory effect on the growth of all tested fungi. Extracts obtained from the same plants had a very similar effect on the growth of tested yeasts, but their effectiveness depended on the type of fungus tested. From the most effective EXs, *Candida tropicalis* was the most resistant to treatment with the EX of *Bidens cerunua* L., with a MIC value of 128 µL/mL. On the contrary, the most sensitive was *C. albicans* (MIC 8 µL/mL for EX and MIC 4 µL/mL for EO). The sensitivity of *C. albicans* to EXs or EOs of this plant species was also demonstrated by **Rybalchenko *et al*. (2010)**. They tested the essential oil from *Bidens cernua* L. by the microdilution method against yeast of the genus *Candida*. According to their results, the effective MIC varied between concentrations of 12.5 to 50 µg/mL depending on the yeast strains used (*Candida albicans* ATCC 885–63 and *Candida albicans* ATCC 18804).

Table 3 Minimum inhibitory concentration (MIC) for tested strains of *Candida* spp.

Plant species	Tested fungi					
	Candida albicans		*Candida glabrata*		*Candida tropicalis*	
	EXs	EOs	EXs	EOs	EXs	EOs
	Minimum inhibitory concentration (MIC) (µL/mL)					
Arctium lappa L.	512	128	-	-	-	-
Bidens cernua L.	8	4	64	32	128	16
Corylus avellana L.	64	32	256	64	256	128
Echinacea purpurea L. (Moench)	128	64	256	128	256	64
Menta piperita L.	16	0.5	128	8	32	4
Trifolium pratense L.	128	64	512	128	256	64
Syzygium aromaticum L.	4	0.25	32	4	16	2
Angelica sinensis (Oliv.) Diels	16	2	128	8	32	8

Legend: - – not tested

Also, **Tomczykova *et al*. (2017)** tested the antifungal effect of EOs from *Bidens tripartita* L. on the growth of *Geotrichum candidum* with MIC values of 3.1 mg/mL. But the strongest effect of this essential oil was found for the bacteria *Neisseria gonorrhoeae* (MIC: 1.56 mg/mL) and *Moraxella catarrhalis* (MIC: 2.07 mg/mL), the weakest effect was found for the yeast *Candida krusei* (MIC > 100 mg/mL). Similarly, authors **Linhares Neto *et al*. (2018)** tested the antifungal activity of *Bidens* spp. on the growth of *C. albicans* and *C. glabrata*. The extract from *Bidens bipinnata* L. showed lower inhibitory effects than the extract from *Bidens cernua* L. tested in our study. But in contrast to our results, in their study, *Bidens bipinnata* L. showed a higher effect on *C. glabrata* than on *C. albicans*. The authors determined the MIC_{90} for *C. albicans* ATCC14057 at a concentration of 64 µg/mL and for *C. glabrata* ATCC2301 at a concentration of 32 µg/mL. *Syzygium aromaticum* L. provided the most effective EX and EO. EX and EO obtained from this plant species inhibited the growth of all tested strains of the genus *Candida* with the lowest MIC values (EX/EO MIC values of 4/0.25 µL/mL for *C. albicans*, EX/EO MIC values of 32/4 µL/mL for *C. glabrata*, and EX/EO MIC values of 16/2 µL/mL for *C. tropicalis*). *C. glabrata* was the most resistant to treatment with this EO. Our results agree with those of **Kim and Lee (2017)** who also tested the inhibitory effect of clove essential oil on the growth of *Candida glabrata*. The MIC values varied between 6.3 - 25 µg/mL in comparison with a control (amphotericin B = MIC 0.5 µg/mL). On the other hand, authors **Pinto *et al*. (2009)** determined the MIC of clove EO for all tested yeasts (*C. albicans*, *C. glabrata*, and *C. tropicalis*) at MIC concentration of 0.64 µL/mL. This fact may be closely related to the composition of individual essential oils used in the studies of other authors and in our study. According to our results, EOs were more effective than EXs in inhibiting the growth of *Candida* species. For example, the authors of **Aiyelaagbe *et al*. (2006)** report that EO obtained from the root of *Jatropha curcas* L. showed a higher inhibitory effect than the clove EX with a MIC value of 0.75 µg/mL on *C. albicans*. Also, EO obtained from leaves of *Rhoeo discolor* L. showed

higher efficacy than clove EX with a MIC value of 1 µg/mL against the strain *C. albicans* ATCC10231 **(García-Varela *et* al., 2015)**.

The results of MIC_{50} and MIC_{90} obtained by probit analysis confirmed the higher efficiency of EOs. The most sensitive strain was *C. albicans*, with the lowest minimum inhibitory concentration able to inhibit 50% of its growth (MIC_{50}) in treatment with both EXs and EOs (Table 4). The most effective EX and EO were those from *Syzygium aromaticum* L., as they were able to inhibit the growth of all tested isolates by the lowest MIC values (MIC_{50} and MIC_{90}) as follows: *C. albicans* (MIC_{50}: 13.51 µL/mL and MIC_{90}: 54.27 µL/mL for EX; MIC_{50}: < 0.125 µL/mL and MIC_{90}: 0.92 µL/mL for EO) < *C. tropicalis* (MIC_{50}: 5.63 µL/mL and MIC_{90}: 8.57 µL/mL for EX; MIC_{50}: 1.06 µL/mL and MIC_{90}: 1.88 µL/mL for EO) < and *C. glabrata* (MIC_{50}: 24.09 µL/mL and MIC_{90}: 27.22 µL/mL for EX; MIC_{50}: 3.01 µL/mL and MIC_{90}: 3.97 µL/mL for EO). According to the probity analysis, the highest MIC_{90} values were found in treatments with *Corylus avellana* L. at a concentration of 334.73 µL/mL for *C. glabrata* and at a MIC_{90} of 314.64 µL/mL for *C. tropicalis*. At the same time, the strain *C. glabrata* appeared to be the most resistant to all tested EXs and EOs, including clove EX and EO.

Table 4 Minimum inhibitory concentrations (MIC_{50} and MIC_{90}) of used EXs/EOs for tested *Candida* spp. evaluated by probit analysis

Plant species	Tested fungi											
	Candida albicans				*Candida glabrata*				*Candida tropicalis*			
	EXs		EOs		EXs		EOs		EXs		EOs	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
AL	512	512	98.96	107.70	NT	NT	NT	NT	NT	NT	NT	NT
BC	20.82	57.72	3.01	3.97	49.05	54.21	24.09	27.22	64.93	73.79	12.02	13.94
CA	48.49	101.09	49.05	54.21	293.52	334.73	49.05	54.21	277.30	314.64	73.61	84.17
EP	92.91	197.87	49.05	54.21	186.99	203.05	98.96	107.70	138.64	157.29	49.05	54.21
MP	27.22	62.79	0.45	1.37	98.96	107.70	4.25	5.74	9.70	14.56	2.77	3.79
TP	92.91	197.87	49.05	54.21	512	512	98.96	107.70	186.99	203.05	49.05	54.21
SA	13.51	54.27	<0.125	0.92	24.09	27.22	3.01	3.97	5.63	8.57	1.06	1.88
AS	27.22	62.79	1.60	2.41	98.96	107.70	6.01	7.28	18.43	22.66	4.19	6.79

Legend: NT – not tested, AL - *Arctium lappa* L., BC - *Bidens cernua* L., CA - *Corylus avellana* L., EP - *Echinacea purpurea* L. (Moench), MP - *Menta piperita* L., TP - *Trifolium pratense* L., SA - *Syzygium aromaticum* L., AS - *Angelica sinensis* (Oliv.) Diels

The chemical composition of used EOs

According to our results obtained from *in vitro* studies of the antifungal activity of EO against the *Candida* strains tested, among the most effective were those EOs characterized by the highest content of eugenol (86.50%) (*Syzygium aromaticum* L.), dihhydrocoumarine (53.20%) (*Angelica sinensis* (Oliv.) Diels), carvone (74.60%) (*Menta piperita* L.), β–trans–ocimene (40.30%), and β–elemene (34.10%) (*Bidens cernua* L.). The content of the major chemical components (above 1%) of the EOs and their authentic standards are summarized in Table 5.

Table 5 The major components of tested EOs analyzed by GC–MS and quantified by GC–FID techniques and the used standards

RI		Component	AE[c]*	AL	AM	BC	CA	EP	MP	TP	SA	AA
932		α–Thujene										1.13
940	[a]	(+)–α–Pinene	8.31			6.06		2.98				
954	[a]	Camphene	3.21									
964		Benzaldehyde								5.89		
980	[a]	(–)–β–Pinene	1.27					1.78				
990		β–Myrcene								4.23		
1006	[a]	α–Phellandrene				2.50		7.89				
1029	[a]	p–Cymene				3.60	3.72	3.74		3.59		
1033	[a]	(R)–(+)–Limonene	1.29					2.35	15.20	3.86		5.36
1035	[a]	Cineol	3.26						1.00			1.32
1043		β–trans–Ocimene				40.30						
1101	[a]	(–)–Linalool	5.72					1.65				
1116		Fenchyl alcohol								3.10		
1105		Nonanal			2.87		2.56			2.72		
1147	[a]	Camphor	2.11									
1158	[a]	(+/–)–citronellal										
1163		Menthone			1.89		3.01					
1169	[a]	(–)–Borneol										
1177	[a]	(–)–Menthol			2.78		2.89		1.30			
1180		Menth–1–en–4–ol			2.32							
1192	[a]	4–Terpineol	1.47					7.81		3.82		
1195		D-Dihydrocarvone								7.25		
1198		α –Terpineol	4.21				19.50		1.20			
1200	[a]	Estragole						2.26				
1204		α–Campholenol					4.74					
1212		Decanal			10.40							
1217		trans–Carveol						4.79				
1227		cis–Carveol						1.01				
1245	[a]	Nerol					21.60					
1247	[a]	(–)–Carvone						1.20	74.60			
1253		Peperitone						1.90				
1259	[a]	Geraniol					2.45					
1287	[a]	Bornyl acetate	3.72									
1296	[a]	Thymol			3.73							
1352		α–Cubebene						1.54				
1357		Citronellol acetate										3.70
1361	[a]	Eugenol			1.56						86.50	
1390		β–Elemene		6.16		34.10						
1407		Methyl eugenol					1.55					
1419	[a]	β–Caryophyllene			11.12	2.70			6.12	15.05	5.00	
1424		Beta-ionone								4.98		
1428		α-Cedrene	2.87									
1435		α–Bergamotene			2.06							
1439		Aromadendrene		16.00								
1452	[a]	α–Caryophyllene				1.80		5.50		3.45	2.00	
1469		α–Humulene			1.51					4.56		

1478		Germacrene D			5.75	1.90		18.20				
1481		γ–Muurolene						2.30				
1490		β–Selinene				2.58						
1494		+)–Ledene										1.70
1496	a	Pentadecane		1.43								
1510	a	Farnesene								10.89		
1513		γ–Cadinene	1.70	1.70	17.15							
1525	a	Myristicin										1.10
1529		Aceteugenol									3.00	
1574	a	Caryophyllene oxide		54.20	5.46	1.89		11.32			1.00	
1589		Spathulenol			1.09		1.12					1.50
1590		Viridiflorol						2.90				
1593		β-Copaen-4α-ol		2.05								
1595		Megastigmatrienone								6.50		
1623		Cedrol	14.30					7.12				
1627		au–Cadinol										1.11
1671		α–Cadinol	1.45		1.99			9.10				
1673		Butylidenephthalide										4.81
1692		6-Butyl-1,4-cycloheptadiene										2.30
1699		α–Bisabolol			1.40							
1725		Penta decanal			1.80							
1741		Dihydrocoumarine										53.20
1728		2,6-Diisopropylnaphthalene								7.61		
1767		β-Costol		4.04								
1900		Nonadecane		1.88								
2000		Eicosane		1.50								
2122		Phytol								7.46		
2400		Tetracosane		1.30								
2600		Hexacosane		2.78								
		Total	**98.56**	**97.42**	**98.65**	**99.15**	**97.45**	**97.34**	**99.70**	**97.23**	**99.60**	**98.23**

Legend: RI – identification based on Kovat's retention indices (HP–5MS capillary column) and mass spectra, a – Identification confirmed by co–injection of authentic standard, c – Relative proportions were calculated in % by dividing individual peak area by total area of all peaks, *AE – *Agrimonia eupatoria* L.; AL – *Arctium lappa* L.; AM – *Arnica montana* L.; BC – *Bidens cernua* L., CA – *Corylus avellana* L., EP – *Echinacea purpurea* L. (Moench), MP – *Menta piperita* L., TP – *Trifolium pratense* L., SA – *Syzygium aromaticum* L., AS – *Angelica sinensis* (Oliv.) Diels

Many other authors have described the high antifungal activities of these EOs and their chemical compositions similar to our results, such as clove EO against fungi of the genus *Aspergillus* **(Císarová *et al*., 2020; Hlebová *et al*., 2021b)**, EOs from *Angelica sinensis* (Oliv.) Diels against *Aspergillus flavus* **(Prakash *et al*., 2015)** and *Candida* spp. **(Nivinskiene *et al*., 2007; Cavaleiro *et al*., 2015; Sowndhararajan *et al*., 2017)**, *Menta piperita* L. EOs against *Candida albicans* **(Samber *et al*., 2015)** and *C. dubliniensis* **(Saharkhiz *et al*., 2012)** and EOs from *Bidens* spp. against *Candida* spp**. (Tomczykowa *et al*., 2011; Tomczykowa *et al*., 2018; Rodin *et al*., 2022)**. The results of the authors **Darvishi *et al*. (2013)** showed that eugenol's mode of action is amino acid permeases inhibition by interfering with two permeases, Tat1p and Gap1p, involved in the dual transport of aromatic and branched-chain amino acids across the yeast cytoplasmic membrane, which may also contribute to amino acid starvation. Carvone, found in *Mentha* spp., inhibits the growth and formation and maintenance of biofilm and the formation of germ tubes in pathogenic yeasts **(Giovana *et al*., 2016)**. β–trans–ocimen, for example, inhibits filamentation, causes membrane damage, and disrupts membrane metabolism in yeast **(Zuzarte *et al*., 2012)**. The other EOs in this study acted differently but usually with a low inhibitory effect. The least effective EOs were those from *Trifolium pratense* L. and *Corylus avellana* L. Their chemical composition can explain the lower efficiency of these EOs. The constituents of *Trifolium pratense* L. were mainly β-caryophyllene (15.05%) and farnesene (10.89%), which were present in relatively small amounts compared to other EOs and their main components, which are responsible for their antifungal activity. These compounds exhibit more potent antibacterial activity, but author **Nogueira Sobrinho *et al*. (2020)** reported that caryophyllene has significant antifungal effects. However, the EOs tested by these authors contained caryophyllene in much greater amounts (39.06%), which may be the reason for the reduced antifungal activity in our study. According to our results, the EO of *Corylus avellana* L. contained nerol (21.60%) as a main component. Different studies have demonstrated that this monoterpene alcohol compound has an excellent antifungal effect **(Wang *et al*., 2020; Hong *et al*., 2023)**. In our study, however, EO and EX with nerol as the main component had no significant inhibitory effect on the tested *Candida* species. The effectiveness of an individual component can be different if it is in a complex mixture (essential oil) with other components because a synergistic or antagonistic effect was also observed between the individual components of the essential oil. Synergistic combinations of components such as eucalyptol in combination with thymol, p-cymene or carvacrol against *Candida* spp. have been confirmed by many authors **(Pina-Vaz *et al*., 2004; da Silva Lima *et al*., 2013**). A synergistic effect is demonstrated by the combination of linalool and geraniol **(Cardoso *et al*., 2016)**. Linalool causes inhibition of cell wall synthesis and increases ion permeability across the cell wall **(Diaz *et al*., 2017)**. Geraniol, in turn, inhibits the formation of biofilms **(Leite *et al*., 2015)**. But for example, the components carvacrol and thymol have the same effect (cause the breakdown of sterols in the cell wall, especially ergosterol) **(da Silva Lima *et al*., 2013)**, and their use in combination has an additive or no effect on the growth of *C. albicans* **(Pina-Vaz *et al*., 2004)**. These combinations show that there must be components with different mechanisms of action for the necessary synergistic effect, as carvacrol and thymol are isomers with the same mechanism of action. Also, synergy is not only influenced by the main components of essential oils (plant extracts) themselves because minor components can also have a decisive role. In contrast, the activity of the main components can be modified (synergistically, antagonistically, or without effect) by these minor molecules **(Sahaf *et al*., 2008)**.

Interaction between tested EOs and EXs

According to the results from MIC determination for individual strains of *Candida* spp., all eight effective EXs and EOs were divided into EXs and EOs with strong and weak antifungal activity as follows: EXs with strong effect can completely inhibit the growth of tested fungi at concentrations from 8 to 32 µL/mL; EXs with weak effect can completely inhibit the growth of tested fungi at concentrations from 64 to 128 µL/mL; EOs with strong effect can inhibit the growth of tested fungi at concentrations from 0.125 to 8 µL/mL; EOs with weak effect can inhibit the growth of tested fungi at concentrations from 16 to 64 µL/mL. Then, the chessboard broth dilution method evaluated the most active concentrations of EXs/EOs (depending on the tested strains). The synergistic effects of EXs and EOs on individual species of the genus *Candida* were mainly observed in combinations of *Syzygium aromaticum* L. with other EXs/EOs. Four mixtures of EXs inhibited the growth of *C. albicans* in combination with each other (strong/weak). The combinations of SA/EP (FICI 0.5), SA/TP (FICI 0.375), AS/EP (FICI 0.375), and MP/TP (FICI 0.5) showed synergistic effects, but the most antagonistic effects of the used EXs in the mixture were recorded for this strain. Antagonistic interactions were observed in treating *C. albicans* with the combination of all strong active EXs and EOs with *Corylus avellana* L. Regarding the antagonistic effects observed in this study, the combination of extracts AS and CA achieved the highest FICI value of 4. As a result, *Corylus avellana* L. was evaluated as the least efficient EX and EO, which had a negative impact on those EXs and EOs that strongly affected the growth of *C. albicans*. This strain was more sensitive to the effect of EO combinations on its growth when compared to the effect of EXs, and the synergistic effect of the EO combinations was observed more frequently in six cases altogether (Table 6).

In the case of the yeast *C. glabrata*, only the clove extract was evaluated as effective, and therefore only three combinations with it were tested (Table 7). All effective (with strong activity) EOs were tested in combination with *Corylus avellana* L. and, similarly to the previous results for *C. albicans*, were evaluated as ineffective. Antagonism was not noted in either case for EOs or EXs, respectively. The most effective combination capable of inhibiting the growth of *C. glabrata* was SA/AS, with a very low FICI value of 0.1875.

Combinations of EXs SA/BC and AS/BC (FICI 0.375 for both combinations) and combinations of EOs SA/EP (FICI 0.5) and MP/EP (FICI 0.375) were synergic against the strain *C. tropicalis* (Table 8). Only the combinations of EXs SA/CA and MP/CA (FICI values of 2 for both combinations) had an antagonistic effect and were not able to inhibit the growth of *C. tropicalis*.

Table 6 Interaction effect of tested EXs/EOs (strong EXs/EOs/weak EXs/EOs) in combination for *Candida albicans*

EXs combination strong/weak	MIC strong EX	MIC strong EX in presence of weak EX	MIC weak EX	MIC weak EX in presence of strong EX	FICI	Outcome
SA/EP	4	1	128	32	0.5	synergism
SA/TP	4	1		16	0.375	synergism
SA/CA	4	4	64	64	2	antagonism
BC/EP	8	6	128	64	1	no effect
BC/TP	8	8		32	1.25	no effect
BC/CA	8	16	64	64	3	antagonism
AS/EP	16	4	128	16	0.375	synergism
AS/TP	16	8		64	1	no effect
AS/CA	16	16	64	128	4	antagonism
MP/EP	16	4	128	32	0.5	synergism
MP/TP	16	4		64	0.75	partial synergism
MP/CA	16	16	64	128	2	antagonism
EOs combination strong/weak	**MIC strong EO**	**MIC strong EO in presence of weak EO**	**MIC weak EO**	**MIC weak EO in presence of strong EO**	**FICI**	**Outcome**
SA/EP	0.25	0.0625	64	8	0.375	synergism
SA/TP	0.25	0.0625			0.1875	synergism
SA/CA	0.25	0.5	32	16	2.25	antagonism
MP/EP	0.5	0.125	64	8	0.375	synergism
MP/TP	0.5	0.25		16	0.75	partial synergism
MP/CA	0.5	0.5	32	32	2	antagonism
AS/EP	2	0.125	64	8	0.125	synergism
AS/TP	2	0.125		32	0.5625	partial synergism
AS/CA	2	2	32	64	2	antagonism
BC/EP	4	0.25	64	8	0.1875	synergism
BC/TP	4	0.5		16	0.375	synergism
BC/CA	4	4	32	32	2	antagonism

Legend: SA – *Syzygium aromaticum* L., EP – *Echinacea purpurea* L. (Moench), TP – *Trifolium pratense* L., CA – *Corylus avellana* L., BC – *Bidens cernua* L., AS – *Angelica sinensis* (Oliv.) Diels, MP – *Menta piperita* L., FICI – fractional inhibitory concentration index

Table 7 Interaction effect of tested EXs/EOs (strong EXs/EOs/weak EXs/EOs) in combination for *Candida glabrata*

EXs combination strong/weak	MIC strong EX	MIC strong EX in presence of weak EX	MIC weak EX	MIC weak EX in presence of strong EX	FICI	Outcome
SA/MP	32	2	128	16	0.75	partial synergism
SA/AS	32	8		64	0.1875	synergism
SA/BC	32	8	64		0.75	partial synergism
EOs combination strong/weak	**MIC strong EO**	**MIC strong EO in presence of weak EO**	**MIC weak EO**	**MIC weak EO in presence of strong EO**	**FICI**	**Outcome**
SA/CA	4	2	64	32	1	no effect
MP/CA	8	4		16		no effect
AS/CA	8	8		32	1.5	no effect
BC/CA	32	16			1	no effect

Legend: SA – *Syzygium aromaticum* L., MP – *Menta piperita* L., AS – *Angelica sinensis* (Oliv.) Diels, BC – *Bidens cernua* L., CA – *Corylus avellana* L., FICI – fractional inhibitory concentration index

Table 8 Interaction effect of tested EXs/EOs (strong EXs/EOs/weak EXs/EOs) in combination for *Candida tropicalis*

EXs combination strong/weak	MIC strong EX	MIC strong EX in presence of weak EX	MIC weak EX	MIC weak EX in presence of strong EX	FICI	Outcome
SA/CA	16	4	128	128	2	antagonism
SA/BC	16	16		16	0.375	synergism
AS/CA	32	8		16	0.75	partial synergism
AS/BC	32	16		32	0.375	synergism
MP/CA	32	16		16	2	antagonism
MP/BC	32	4		64	1	no effect
EOs combination strong/weak	**MIC strong EO**	**MIC strong EO in presence of weak EO**	**MIC weak EO**	**MIC weak EO in presence of strong EO**	**FICI**	**Outcome**
SA/EP	2	0.125	64	16	0.5	synergism
SA/TP	2	0.5		64	1.0625	no effect
MP/EP	4	0.5		16	0.375	synergism
MP/TP	4	2		32	1	no effect
AS/EP	8	4		64	1.5	no effect
AS/TP	8	4		64	1.5	no effect
BC/EP	16	8		32	1	no effect
BC/TP	16	16		32	1.5	no effect

Legend: SA – *Syzygium aromaticum* L., CA – *Corylus avellana* L., BC – *Bidens cernua* L., AS – *Angelica sinensis* (Oliv.) Diels, MP – *Mentha piperita* L., FICI – fractional inhibitory concentration index

Some of the EXs and EOs tested in this study have never been studied as antifungal agents against yeasts or fungi, so it was not possible to compare our results with any other authors. Our results showed that the EX/EO of *Syzygium aromaticum* L. was the most effective. The excellent effects of clove EO have been recorded in many previous and other studies **(Císarová *et al.*, 2020; da Silva Campelo *et al.*, 2021; Hlebová *et al.*, 2021b; Mahdi *et al.*, 2021).** Exceptional antifungal effects of this EO on the growth of fungi of the genus *Aspergillus*, both alone and in combination with other EOs, are described in our previous study **(Hlebová *et al.*, 2021a)**. Also, **Hassan *et al.* (2020)** reported that combining EOs (cumin and caraway EOs) with clove EO synergized in inhibiting *C. albicans* and *A. niger* growth. In particular, eugenol, the main component of clove EO, exhibits a strong synergistic effect. Therefore, an advantageous combination can be developed for EOs with antifungal agents to treat *Candida* spp., regardless of their sensitivity to individual antifungal agents **(Khan *et al.*, 2012)**.

The fungicidal effect of EXs and EOs reduces the necessary dose of antifungal drugs to act against yeast. Moreover, the synergistic effect of EXs and EOs with

commercially available antifungals was also observed. Fluconazole, a hydrophilic azole, does not bind to or incorporate into the cytoplasmic membrane but must enter the cell to act on it. The synergistic combination of fluconazole and amphotericin B with EOs can be explained as EOs promoting the effect of antifungal drugs, especially on the cell wall, plasma membrane, and other membrane structures of yeast cells **(Pinto *et al*., 2009)**. EOs can damage the cell wall and cytoplasmic membrane, which, in a synergistic effect with fluconazole, facilitates their entry into the cell and leads to a more significant effect on the inhibition of ergosterol biosynthesis. Such a synergistic combination can change the fungistatic effect of fluconazole into a fungicidal effect. In the case of amphotericin B, its toxicity depends on the dose used; in a synergistic combination with EOs, the toxicity will be reduced with the same effect. These fluconazole or amphotericin B combinations with EOs are comparable with echinocandins, targeting the enzyme 1,3-β-glucan synthase and disrupting cell wall synthesis **(Onyewu *et al*., 2007)**. Furthermore, the potent fungicidal activity of EXs and EOs against isolates resistant to azoles and amphotericin B suggests that EXs and EOs are effective against the strategies or adaptive resistance mechanisms exhibited by *Candida* isolates against these antifungal drugs **(Khan and Ahmad, 2012)**.

CONCLUSION

In summary, the antifungal activities of mixtures of EXs and EOs against all strains of the genus *Candida* tested in this study were generally superior to those of single EXs and EOs. The EX and EO of *Syzygium aromaticum* L. Clove EX showed the greatest inhibitory effect, and EO exhibited synergistic action capable of inhibiting the growth of all three tested species when combined with other tested EXs and EOs. Further therapeutic research is essential to evaluate the synergism of natural substance mixtures or combinations of natural substances with already–used antifungals. However, there are also potential limits because it is not always possible to combine EOs with all antifungals and it also depends on the overall health of the patient (allergies to individual plants, etc.). Therefore, the recommendation for the future evaluations is that the synergistic effect of EXs, EOs, or its components could be tested in a triple combination among themselves or with commercially available antifungals, e.g., amphotericin B, which has already demonstrated a high inhibitory potential in a double combination, for example, with clove EO, against resistant *Candida* species. Synergistic interactions between EXs/EOs and commercial antifungals may enable the use of reduced antifungal doses while minimizing side effects.

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