ORIGINAL ARTICLE

Journal of Pre-Clinical and Clinical Research 2021, Vol 15, No 1, 15-19
www.jpccr.eu

Isobolographic *in vitro* interactions of fluconazole with citrus essential oils against *Cladosporium cladosporioides*

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of article

Wróblewska-Łuczka P. Isobolographic *in vitro* interactions of fluconazole with citrus essential oils against *Cladosporium cladosporioides*. J Pre-Clin Clin Res. 2021; 15(1): 15–19. doi: 10.26444/jpccr/132014

Abstract

Introduction. Cladosporium is one of the most abundant genera of environmental fungi worldwide and a very common respiratory allergen. To-date, many C. cladosporoides infections have been identified. Risk factors for C. cladosporioides infection are primarily injuries, metabolic disorders, organ transplantation and autoimmune diseases, among others.

Objective. The aim of the study was to assess the type of pharmacodynamic interactions between fluconazole and some selected essential oils: orange, mandarin, lemon and grapefruit, in an *in vitro* study against C. cladosporioides.

Materials and method. Experiments were carried out using the plate cultivation method. Fluconazole was tested against C. cladosporioides at concentrations ranging from 0.05–3.3 mg / ml, and the activity of essential oils added to PDA medium at concentrations ranging from 1–30%. The dose-effect curves for the collected results were determined with by the log-probit method. Isobolographic analysis of the results allowed determining the type of interactions between fluconazole and the tested essential oils.

Results. Lemon essential oil was the most active, and in a concentration of 1% it inhibited the growth of C. cladosporioides by 21%. Isobolographic analysis showed that the combination of fluconazole with orange and grapefruit essential oil had an additive interaction, and with mandarin and lemon – an additive interaction with a tendency to synergy in the plate culture test for C. cladosporioides.

Conclusions. The use of isobolographic analysis can contribute to the introduction of natural substances with the desired activities into the pharmacotherapy of many infections and diseases. The use of natural substances can also help to reduce the number of side-effects caused by conventional and standard therapies.

Key words

essential oils, moulds, isobolographic analysis, fluconazole, Cladosporium

INTRODUCTION

Cladosporium is one of the most abundant genera of environmental fungi worldwide and *Cladosporium herbarum* is one of the most common species that is isolated. Like *Alternaria*, *Cladosporium* is widely recognized as a fungus present in the outside air [1], but is often also found indoors [2]. The clinical significance of *Cladosporium* is related to the fact that this fungus is a respiratory allergen. Data from ECHRS (European Community Respiratory Health Survey) indicate that the incidence of positive skin tests for *Cladosporium* is 1.7% (ranging from 0–11.9%) [3]. The threshold concentration for the induction of allergic symptoms by *Cladosporium* was estimated to be 3,000 spores/m^3 air [4, 5].

The genus *Cladosporium*, first identified in 1815, now comprises around 500 species [6, 7]. *Cladosporium* species are most commonly found in outdoor and indoor environments, on decayed organic substrates, and are considered important food contaminants [8, 9]. These fungi can use a variety of growing substrates, such as wood, plants, food, soil, straw and textiles [10]. The most frequently isolated species are *Cladosporium sphaerospermum*, *Cladosporium cladosporioides*, *Cladosporium herbarium* and *Cladosporium elatum* [6]. Many species of *Cladosporium* fungi are capable of producing certain secondary metabolites, such as antibiotics, which are inhibitors of *Bacillus subtilis*, *Escherichia coli* and *Candida albicans* [11, 12].

Cladosporium cladosporioides is a rare, although worldwide, infectious pathogen. To- date, 21 cases of human fungal infection have been reported: six in India, five in China, three in America, and one each in The Netherlands, Spain, Portugal, Germany, Malaysia, Brazil and Nigeria [13]. The risk factors for *C. cladosporioides* infection are primarily injuries (especially of the eyes), metabolic disorders, organ transplantation, autoimmune diseases, human immunodeficiency virus infections, and mycobacterial tuberculosis infections. The types of infection caused by C. cladosporioides include infection of the brain, lung, pancreas, eye, nail and subcutaneous infection. Clinical symptoms of *C. cladosporioides* infections include mainly papules, nodules and cysts, most often located on the limbs [14], less often on the scalp, or as cystic lesions on the face [13]. There is no standard treatment for infections caused by *C. cladosporioides*. Treatment includes antifungal therapy and immune enhancement [15]. Often, an orally administered azole drug, e.g. fluconazole, itraconazole) is used as an antifungal drug, which has successfully treated many patients, especially those with immunodeficiency [13, 16].

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Received: 15.11.2020; accepted: 29.12.2020; first published: 18.01.2021

Essential oils have been known for a long time. They are used for food, cosmetic and medicinal purposes. Essential oils have many pharmacological properties depending on the main chemical components. Essential oils have a diuretic effect (parsley and juniper oils), are used as a base for expectorant syrups (anise oil, fennel oil or thyme oil), have strong anti-inflammatory properties (chamomile oil), choleretic properties (peppermint oil) and have a calming effect (valerian oil, melissa oil) [17]. The bacteriostatic and fungistatic activities of essential oils are used in the pharmaceutical, food and cosmetics industries [18].

OBJECTIVES

The aim of the study was to assess the nature of the pharmacodynamic interactions between fluconazole and the essential oils: orange, mandarin, lemon and grapefruit against *Cladosporium cladosporioides* in *in vitro* studies.

MATERIALS AND METHOD

The research was carried out using the method of plate cultivation on PDA – Potato Dextrose Agar (Biocorp) medium with 1% Tween 20. Cultures were carried out for eight days at 37 °C. Fluconazole was tested against *Cladosporium cladosporioides* at concentrations ranging from 0.05–3.3 mg/ml and the activity of essential oils added to PDA medium at concentrations ranging from 1–30%. The following essential oils were used in the research: orange (*Citrus aurantium*), lemon (*Citrus limon*), mandarin (*Citrus reticulata*) and grapefruit *(Citrus paradise*). Commercially available essential oils (Avicenna-Oil, Poland) were used for the research. The test substance was added to the medium, PDA with 1% Tween 20, in an appropriate amount. Each of the assays was performed in triplicate. After eight days of growth, the size of the colony was measured in comparison with the control, which allowed determination of the percentage of inhibition of fungal growth caused by the addition of fluconazole or essential oils to the medium [19, 20]. The obtained results were used to determine the 'dose-effect' curve of the dependence of the concentration of the tested substances or their mixtures in the medium to the percentage of fungal growth inhibition, according to the log-probit method (Litchfield & Wilcoxon, 1949) [21]. The IC_{50} (median inhibitory concentration, 50% inhibitory effect on the growth of *C.cladosporioides*) doses of the test substances were determined. The test for parallelism of concentration-response effect lines for fluconazole and each of the tested essential oils was performed. This had an impact on the appearance of the additivity line on the isobologram. The effect of a mixture of fluconazole and essential oils in a constant ratio of 1:1 on *Cladosporium cladosporioides* was also assessed by the plate culture method. Isobolographic analysis of the obtained results was performed [22], which allowed determination of the interaction between fluconazole and the tested essential oils.

RESULTS

Based on the plate culture tests performed for Cladosporium cladosporioides, it was shown that fluconazole in the medium at a concentration of up to 0.4 mg / ml does not cause any inhibition of fungal growth, while the concentration of 3.3 mg/ml causes growth inhibition at the level of 76% (Fig. 1).

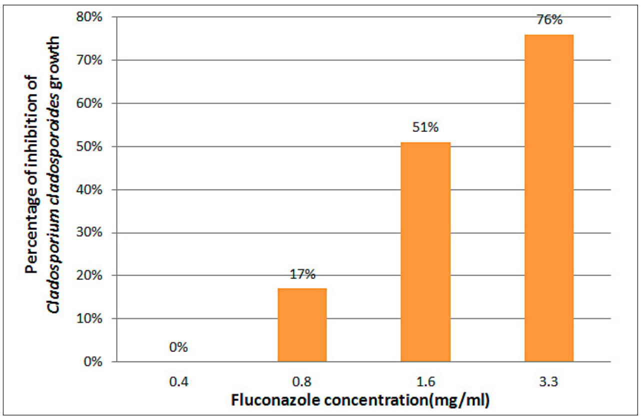

Figure 1. Fluconazole concentration (mg/ml) versus percentage inhibition of *Cladosporium cladosporioides* growth

In the case of essential oils, it was determined that the orange and grapefruit essential oils did not show growth inhibition up to a concentration of 1%, while at a concentration of 10% it completely inhibited the growth of *Cladosporium cladosporioides*. The concentration of mandarin essential oil in the medium to 1% did not cause any inhibition of the growth of *Cladosporium cladosporioides*, while the concentration of 30%, it caused a complete inhibition of the growth of this fungus. Lemon essential oil was the most active and already at a concentration of 1% it inhibited the growth of *Cladosporium cladosporioides* by 21%. On the other hand, the concentration of 15% and more of lemon oil in the medium caused complete inhibition of the growth of *Cladosporium cladosporioides* (Fig. 2).

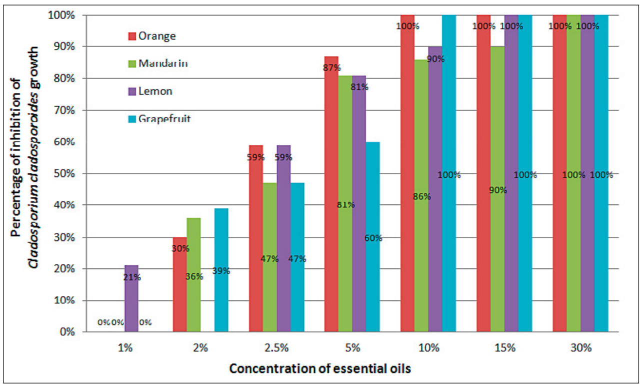

Figure 2. Dependence of the concentration of the studied essential oils in the medium on the percentage of growth inhibition of *Cladosporium cladosporioides*.

Based on the obtained results, using the logarithmic-probit method according to Litchfield and Wilcoxon (1949) [21], graphs were drawn for the 'dose-effect' relationship of the tested essential oils, fluconazole and their 1:1 mixture against *Cladosporium cladosporioides* (Fig. 3). This allowed the determination of IC_{50} doses for individual tested substances (Tab. 1). Lemon oil proved to be the most active against *C.*

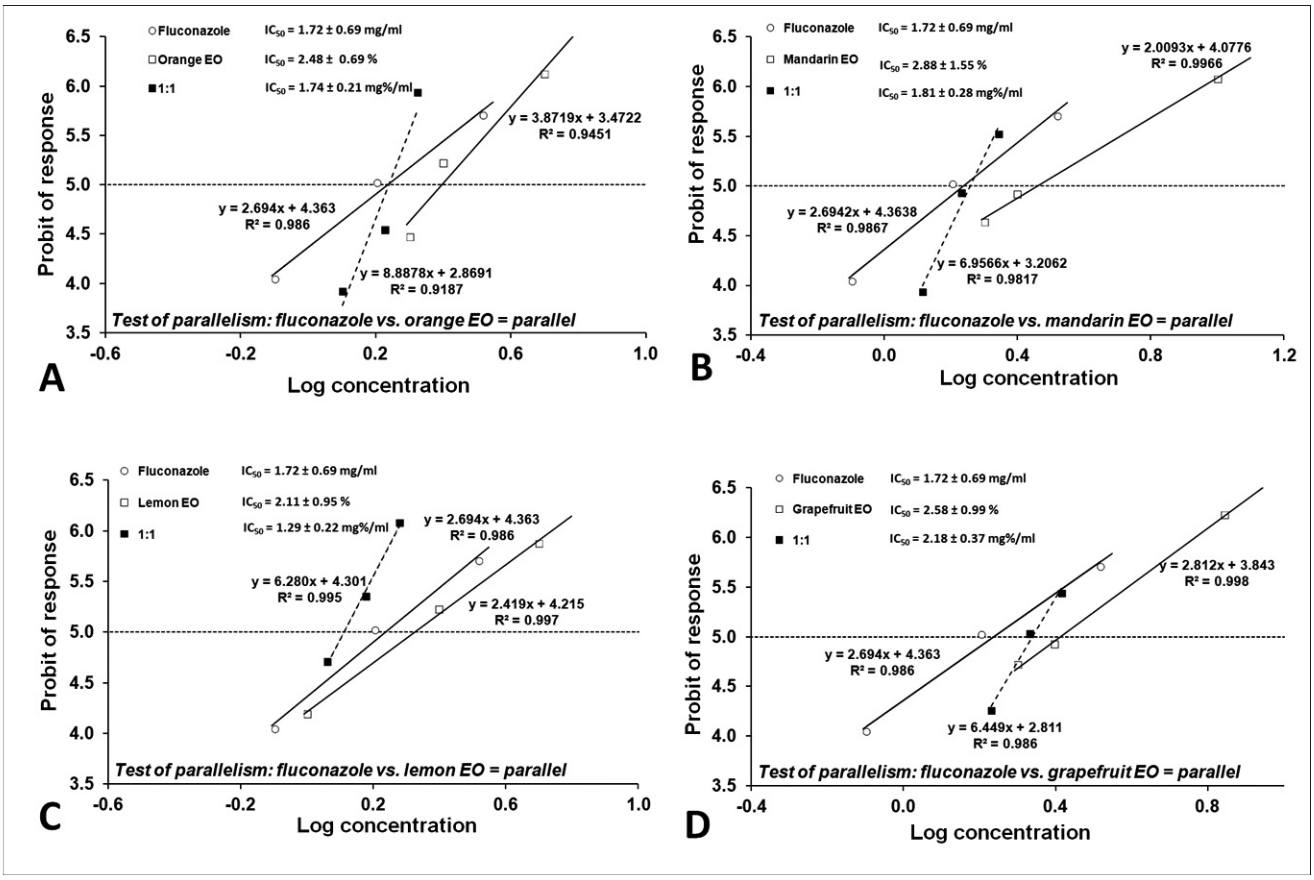

Figure 3. Log-probit dependence of the concentrations of fluconazole, essential oil and their combinations on the inhibition of *Cladosporium cladosporioides* colony growth

cladosporoides and had the lowest IC_{50} value, compared to the other tested citrus essential oils.

Table 1. IC_{50} values of the active substances tested against *Cladosporium cladosporioides*

Substance	IC_{50} of test substance
Fluconazole	IC_{50} = 1.72±0.69 mg/ml
Orange essential oil	IC_{50} = 2.48±0.69 %
Mandarin essential oil	IC_{50} = 2.88±1.55 %
Lemon essential oil	IC_{50} = 2.11±0.95 %
Grapefruit essential oil	IC_{50} = 2.58±0.99 %

Concentrations of test substances were converted to decimal logarithms, and inhibition of *Cladosporium cladosporoides* colony growth was converted into probit responses. The linear dose-effect equations for fluconazole, orange (A), mandarin (B), lemon (C), and grapefruit (D) essential oils and their combinations in a constant 1: 1 ratio are shown in the Figure, where: x – decimal logarithm of the concentration of test substances, y – response probit and R^2 – coefficient of determination. From the logarithmic-probit equations, the values of the median concentrations inhibiting the growth of fungal colonies (IC50 ± S.E.) for fluconazole and essential oils and their combinations in a constant concentration ratio of 1:1 were calculated.

On the basis of the plate culture tests performed for *Cladosporium cladosporioides*, IC_{50} values were determined for the mixture of fluconazole with the studied essential oils. Isobolograms were drawn showing the interactions of the studied essential oils with fluconazole in a constant 1: 1 ratio (Fig. 4).

Isobolograms showing additive with a tendency to synergy interactions between fluconazole and mandarin (B) and lemon (C) essential oils. Isobolograms showing additive interactions between fluconazole and orange (A) and grapefruit (D) essential oils. The median inhibitory concentrations (IC_{50}) for fluconazole and the tested essential oils are plotted on the X- and Y-axes, respectively. The solid lines on the X and Y axes represent the S.E. for the IC_{50} values for the studied drugs, when administered alone. For collateral concentration-response relationships between fluconazole and the studied essential oils, the isobole represents the straight diagonal line connecting the IC_{50} values. The dotted line starting from the point (0, 0) corresponds to the fixed-ratio of 1:1 for the combination of fluconazole and the tested essential oils. Point A depicts the theoretically calculated $IC_{50\,add}$ value for isobole of additivity. The point M represents the experimentally-derived $IC_{50\,exp}$ value for total dose of the mixture expressed as proportions of fluconazole and each of the tested essential oils that produced a 50% inhibitory growth of *Cladosporium cladosporoides,* as investigated in *in vitro* studies.

Isobolographic analysis showed that the combination of fluconazole with orange and grapefruit essential oils had an additive interaction, and with mandarin and lemon – an additive interaction with a tendency to synergy in plate culture tests for *Cladosporium cladosporioides*.

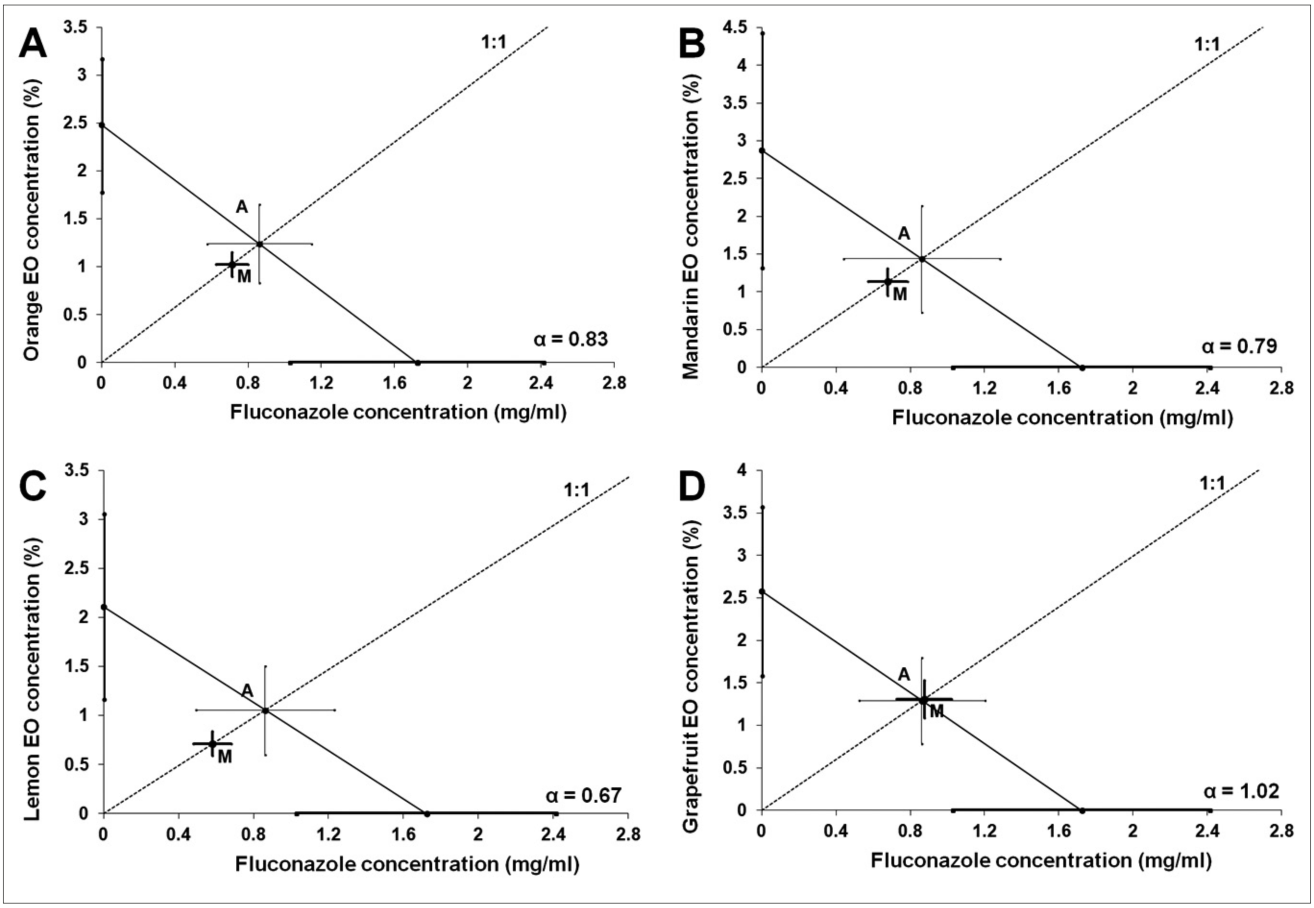

Figure 4. Isobolograms for interactions of fluconazole with the essential oil against *Cladosporium cladosporioides*

DISCUSSION AND CONCLUSIONS

For a very long time, compounds of natural origin were the only medicinal substances available to man, and still play an important role, especially in self-healing processes. The use of herbs and plants as medicines and nutraceuticals is now an important area of research. About 80% of the world's human population believes in the health benefits of plants [23]. Essential oils are characterized by numerous biological properties. The essential oils of citrus have antibacterial and antifungal properties [24, 25], but also have an effect on the central nervous system, demonstrating an anxiolytic effect [26, 27].

Yew et al. have found that the MIC concentration of fluconazole against many moulds depending on the strain. The researchers assessed the MIC value using the ready-made 'Etest' test by Biomerieux. The IC_{50} dose of fluconazole determined in this study can be compared with the minimum concentration inhibiting the growth of microorganisms in 50% is slightly higher than in the quoted study, which may be due to methodological differences, among others: the use of a ready-made test and the *C. cladosporioides* strain [28]. The same limitations apply to the evaluation of fungal growth inhibition by essential oils. The test methodology and the type of essential oils used have a great influence on the result [29]. Note that although standard antifungal susceptibility tests are currently available, information from *in vitro* studies is limited as it only provides a static measure of antimicrobial activity in a specific medium. Biological fluids, such as human serum and urine, may have a different effect on the pharmacodynamics of the active substance.

It is worth emphasizing that both fluconazole and essential oils inhibit cytochrome P450 (CYP) 3A4 enzymes in fungi; therefore, their combination is justified. CYP enzymes are responsible for the synthesis of ergosterol (the main sterol of the fungal cell membrane), which causes changes in the fluidity of the cell membrane, thus inhibiting fungal replication [30, 31].

Isobographic analysis is mainly used to evaluate drug interactions in animal studies. In *in vitro* microbiological studies it has only been used a few times, mainly to assess the interaction between antibiotics in the treatment of *Mycobacterium abscessus* and *Mycobacterium avium infections* [*32*], the interaction of chitosan and silver nanoparticles in inhibiting *Staphylococcus ureus*, *Pseudomonas aeruginosa* [*33*], and the interaction of antibiotics in the inactivation of *Aspergillus fumigatus* [34].

CONCLUSIONS

The use of isobolographic analysis can contribute to the introduction of natural substances with the desired activities into the pharmacotherapy of many infections and diseases. The use of natural substances can also help to reduce the number of side-effects caused by conventional and standard therapies.

The promising results of the conducted *in vitro* studies encourage and justify the need for further research and analyze, which, consequently, may contribute to the development of new methods of treating fungal infections with the use of known antifungal drugs and substances of natural origin.

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