



# Additivity interactions between fluconazole and citrus essential oils to *Aspergillus fumigatus*

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## Abstract

**Introduction.** *Aspergillus fumigatus* is the most common pathogen causing allergic bronchopulmonary mycosis. The pathogenic capacity of *Aspergillus fumigatus* is related to its thermal tolerance and the small size of the spores which enables transfer to the respiratory tract. In the case of fungal diseases, their treatment is based on fungicidal antibiotics, such as fluconazole. Due to the growing problem of drug resistance, new therapeutic solutions are sought, especially of natural origin. Essential oils, due to their anti-bacterial, anti-fungal, anti-inflammatory and immunostimulatory properties, constitute interesting research material in the fight against mould.

**Objective.** The aim of the study was to assess the type of pharmacodynamic interactions between fluconazole and selected essential oils: lemon, orange, tangerine, and grapefruit in an *in vitro* study against *Aspergillus fumigatus*. Isobolographic analysis of the results allowed determining the type of interactions between fluconazole and the tested essential oils.

**Results.** According to the research results, a  $IC_{50}$  dose of fluconazole versus *Aspergillus fumigatus*  $IC_{50} = 1.87 \pm 0.88$  mg/ml. The most active essential oil was lemon oil, which at the concentration of 4% in medium completely inhibited the growth of *Aspergillus fumigatus*. Tangerine essential oil is the least active against *A. fumigatus*. Isobolographic analysis of the interactions between fluconazole and essential oils showed additive interactions for the combination of fluconazole with lemon, orange and grapefruit oils, and an additive interaction with a tendency to synergism for the combination of fluconazole with tangerine oil.

**Conclusions.** Isobolographic analysis can contribute to the introduction of natural substances into the therapy of many diseases.

## Key words

*Aspergillus*, essential oils, moulds, isobolographic analysis, fluconazole

## Abbreviations

EO – essential oil;  $IC_{50}$  – median inhibitory concentration;  $IC_{50\text{ mix}}$  – median inhibitory concentration for the mixture of the tested substances; PDA – Potato Dextrose Agar medium

## INTRODUCTION AND OBJECTIVE

*Aspergillus fumigatus* is the most common pathogen causing allergic bronchopulmonary mycosis. Moreover, it is often isolated from the respiratory tract in patients with asthma who do not meet the criteria for allergic bronchopulmonary aspergillosis [1, 2], and from patients with respiratory diseases other than asthma, including cystic fibrosis [3, 4], chronic obstructive pulmonary disease [5], tuberculosis associated with fibrosis [6], and sometimes in the respiratory tract of healthy people [7]. The pathogenic capacity of *Aspergillus fumigatus* is related to its thermal tolerance, which allows it to grow at the temperature of the human body, and the small size of the spores (about 2–3  $\mu\text{m}$ ), which enables transfer to the respiratory tract [8]. *Aspergillus fumigatus* causes aspergillosis in immunocompromised patients. Invasive aspergillosis is still associated with a high mortality rate, ranging from 30% – 90% of patients [9, 10]. It is estimated that *Aspergillus fumigatus* is the etiological factor of various forms of aspergillosis in over 200,000 patients annually [11].

In the case of fungal diseases, their treatment is based on fungicidal antibiotics, such as fluconazole [12, 13]. Due to the growing problem of drug resistance, new therapeutic solutions are sought for, especially those of natural origin. Essential oils, due to their anti-bacterial, anti-fungal, anti-inflammatory and immunostimulatory properties, constitute interesting research material in the fight against mould [14–18].

For a very long time, compounds of natural origin were the only medicinal substances available to humans. They 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 population believe in the health benefits of plants [19, 20]. Active substances of natural origin are a rich source of compounds with different chemical structures, often showing different biological activities. They are substances showing a high affinity for binding with, e.g. enzymes or receptors found in living organisms. With time, drugs of natural origin have been replaced by their semi-synthetic and synthetic derivatives. Currently, new drugs with interesting chemical structures and previously unknown mechanisms of action are sought. The introduction of new drugs for use is a long-term process due to the duration of preclinical and clinical trials. The search for new drugs results from the depletion of current treatment

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options, including bacterial and fungal infections, especially in the case of multi-drug resistant strains [21, 22]. Antibiotic resistance is both a medical and an economic problem, caused by the abuse of many antimicrobial drugs. Essential oils may be an effective alternative to antibiotics when fighting drug-resistant microorganisms [17, 18, 23]. Many essential oils and plant extracts have strong anti-microbial properties. Due to the increasing popularity of natural medicines, as well as the market demand for such products, it seems very important to study the properties of plants and their secondary metabolites, including essential oils [24, 25].

Isobographic analysis is mainly used to evaluate drug interactions in animal studies. In *in vitro* microbiological studies it has been used only several times, mainly to assess the interaction of fluconazole with citrus essential oils against *Cladosporium cladosporioides* [26], the interaction of antibiotics in the treatment of *Aspergillus fumigatus* [27–30], or the assessment of interactions between antibiotics in the treatment of mycobacterial infections *Mycobacterium* [31].

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

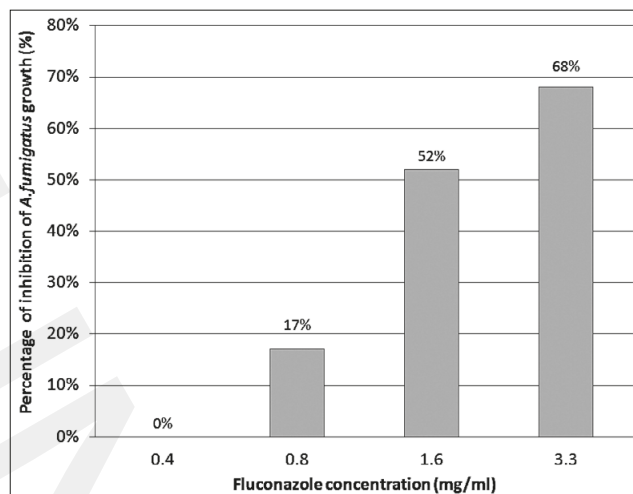
## MATERIALS AND METHOD

**Plate tests.** 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 8 days at 37 °C. Fluconazole was tested against *Aspergillus fumigatus* 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: lemon (*Citrus limon*), orange (*Citrus aurantium*), tangerine (*Citrus reticulata*) and grapefruit (*Citrus paradisi*). 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 8 days of growth, the size of the colony was measured in comparison with the control, which allowed to determine the percentage of inhibition of fungal growth caused by the addition of fluconazole or essential oils to the medium [32,33].

**Isobolographic analysis.** 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, according to Litchfield and Wilcoxon [34]. The  $IC_{50}$  (median inhibitory concentration, 50% inhibitory effect on the growth of *A.fumigatus*) 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 isoblogram. The effect of a mixture of fluconazole and essential oils in a constant ratio of 1:1 on *Aspergillus fumigatus* was also assessed by the plate culture method. The isobolographic analysis of the obtained results was performed [35–37], which allowed determination of the interaction between fluconazole and the tested essential oils.

## RESULTS

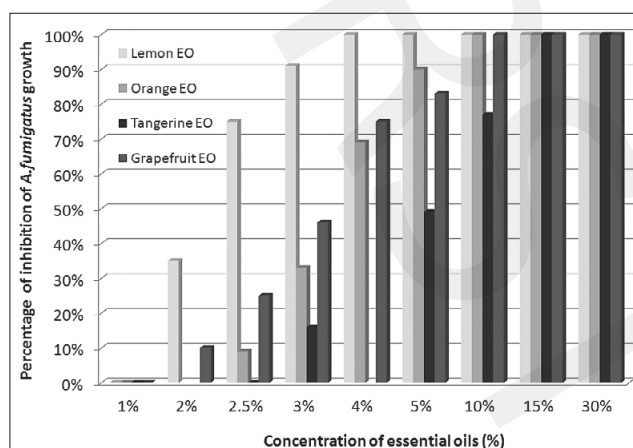
Based on the plate tests for *Aspergillus fumigatus*, it was shown that fluconazole in the medium at a concentration of up to 0.4 mg/ml did not cause any inhibition of fungal growth, while the concentration of 3.3 mg/ml caused a growth inhibition of 68% (Fig. 1).



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

In the case of essential oils, it was determined that none of the tested oils inhibited the growth of *A. fumigatus* at a concentration of 1%. Lemon essential oil was the most active at a concentration of 4% and higher in the medium, it completely inhibited the growth of *Aspergillus fumigatus*. Orange and grapefruit essential oils showed weaker activity than lemon oil, at a concentration of 10% they completely inhibited the growth of *A. fumigatus*. Tangerine essential oil was the weakest in relation to *A. fumigatus*, the concentration of 15% of the oil in the medium caused a complete inhibition of the growth of this fungus (Fig. 2).

Based on the obtained results, using the log-probit method according to Litchfield and Wilcoxon (1949) [34], graphs were drawn for the ‘dose-effect’ relationship of the tested essential oils, fluconazole and their 1:1 mixture against *Aspergillus fumigatus* (Fig. 3).  $IC_{50}$  doses were determined for fluconazole and the tested essential oils against *A. fumigatus* (Tab. 1).

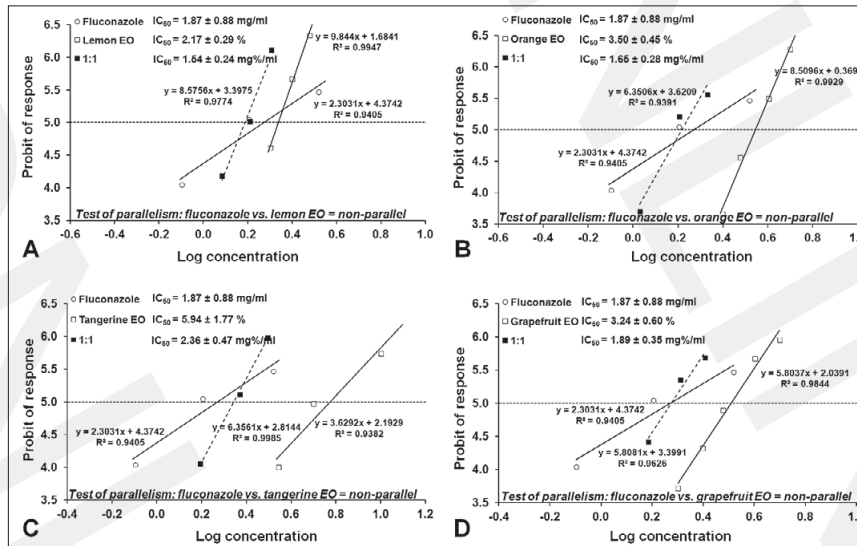
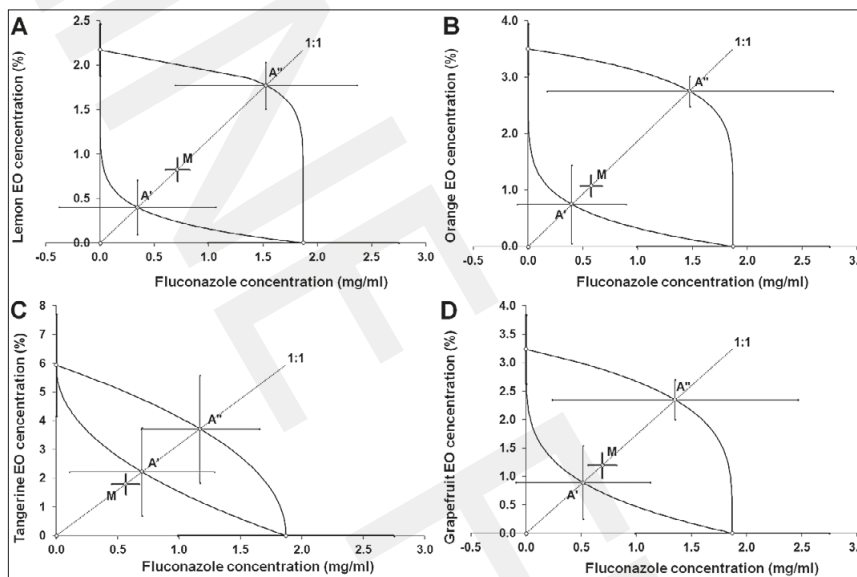


**Figure 2.** Dependence of the concentration of the studied essential oils in the medium on the percentage of growth inhibition of *Aspergillus fumigatus*

**Table 1.** Antifungal effect of fluconazole and 4 essential oils: lemon, orange, tangerinen and grapefruit administrated singly in the *Aspergillus fumigatus*

Substance	IC <sub>50</sub> ± S.E.
Fluconazole	IC <sub>50</sub> = 1,87±0,88 mg/ml
Lemon EO	IC <sub>50</sub> = 2,17±0,29 %
Orange EO	IC <sub>50</sub> = 3,50±0,45 %
Tangerine EO	IC <sub>50</sub> = 5,94±1,77 %
Grapefruit EO	IC <sub>50</sub> = 3,24±0,60 %

On the basis of plate tests for *Aspergillus fumigatus*, IC<sub>50</sub> values were determined for a mixture of the tested essential oil and fluconazole in a constant dose ratio of 1:1 and for lemon oil: IC<sub>50 mix</sub> = 1.54 ± 0.24 mg%/ml, for orange oil: IC<sub>50 mix</sub> = 1.65 ± 0.28 mg%/ml, for tangerine oil: IC<sub>50 mix</sub> = 2.36 ± 0.47 mg%/ml and grapefruit: IC<sub>50 mix</sub> = 1.89 ± 0.35 mg%/ml. Isobolograms were created showing the interactions of the studied essential oils with fluconazole in a constant 1:1 ratio. Isobolographic analysis showed that the combination of fluconazole with orange, lemon and grapefruit essential oil had an additive

**Figure 3.** Log-probit concentration-response of the concentrations of fluconazole, essential oil and their combinations on the inhibition of *Aspergillus fumigatus* colony growth. Log-probit concentration-response relationship lines for fluconazole and 4 tested essential oils administered alone and in combination at the fixed-ratio of 1:1, illustrating the antifungal effects of the drugs in *Aspergillus fumigatus* measured *in vitro* by plate method**Figure 4.** Isobolograms showing interactions between fluconazole and essential oils in relation to *Aspergillus fumigatus*. Isobolograms showing additive interactions between fluconazole and tested essential oils for *Aspergillus fumigatus*. The median inhibitory concentrations (IC<sub>50</sub>) for fluconazole and the tested essential oils (lemon (A), orange (B), tangerine (C) and grapefruit (D)) 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<sub>50</sub> values for the studied drugs, when administered alone. The lower and upper isoboles of additivity represent the curves connecting the IC<sub>50</sub> values for fluconazole and the tested essential oils administered alone, if their concentration-response relationships were non-parallel. The dotted line starting from the point (0, 0) corresponds to the fixed-ratio of 1:1 for the combination of fluconazole with each of the tested essential oils. The points A' and A'' depict the theoretically calculated IC<sub>50 add</sub> values for both, lower and upper isoboles of additivity. The point M represents the experimentally-derived IC<sub>50 exp</sub> value for total dose of the mixture expressed as proportions of fluconazole and each of the tested essential oils that produced a 50% antifungal effect in *Aspergillus fumigatus*, as measured *in vitro* by plate tests



interaction, and with tangerine essential oil – an additive interaction with a tendency to synergism in plate tests for *Aspergillus fumigatus* (Fig. 4).

## DISCUSSION

Fluconazole is used in some experimental studies as a standard anti-fungal drug against, among others: *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata* [38–40]. The minimum concentration of fluconazole that inhibited the growth of *Aspergillus fumigatus* in 50% was 16 µg/ml [41]. However, the minimum concentration inhibiting the growth of 80% of *Aspergillus fumigatus* for fluconazole was from 64 µg/ml [42]. The results presented in this study showed that the IC<sub>50</sub> dose of fluconazole, which can be compared with the minimum concentration inhibiting the growth of microorganisms in 50%, is slightly higher than in the presented studies, which may be due to methodological differences, e.g. the use of microtiter plates [41, 42], or shortening breeding time of 5–7 days [42]. According to the results of the current research, the IC<sub>50</sub> dose of fluconazole versus *Aspergillus fumigatus* IC<sub>50</sub> = 1.87±0,88 mg/ml.

The action of grapefruit oil was species-specific. Lower MIC concentrations were observed for yeasts compared to mould fungi. *Candida* species are more sensitive to the action of grapefruit essential oil compared to *Aspergillus* moulds. Interestingly, grapefruit essential oil showed a stronger antifungal activity compared to pure limonene, which indicated a synergistic effect of all components of the oil. The minimum inhibitory concentration of grapefruit essential oil was determined to be 100 mg/l after a 2-day incubation of *Aspergillus fumigatus* [43]. Tangerine essential oil exhibited a wide anti-fungal spectrum inhibiting the growth of *Aspergillus fumigatus*, *Aspergillus terreus*, *Alternaria alternata*, *Fusarium oxysporum*, *Penicillium italicum* and *Trichoderma viride* at 750 ppm of oil in PDA medium [44]. After 14 days of incubation, *Aspergillus flavus* growth was inhibited to 18.70% in response to lemon essential oil, and 5.92% to orange essential oil. The essential oils showed a potential inhibitory activity against toxic fungi and inhibited the production of mycotoxins, especially aflatoxin B1 [45]. In other studies, bergamot oil (from the citrus-bergamot orange) was found to be the most effective, inhibiting the growth of *Aspergillus flavus* by 76.51% at a concentration of 5% of the oil in medium. Other active oils were: bitter orange and sweet orange oil (inhibiting mycelium growth in approx. 60% at 5% oil concentration), and then lemon oil (inhibiting mycelium growth in approx. 55% at 5% oil concentration). Tangerine essential oil showed the lowest percentage of mycelial inhibition compared to other citrus essential oils. The observed differences in anti-fungal activity among citrus essential oils may be due to differences in chemical composition, although limonene can be considered the main inhibitory component present in citrus essential oils [32].

The presented research also confirms that tangerine essential oil is the least active against *Aspergillus fumigatus*. The most active was lemon oil, which at the concentration of 4% completely inhibited the growth of *Aspergillus fumigatus*. The differences may result from species differences between the fungi used in the research and those indicated by other authors, and from differences in the composition of the tested essential oils.

The isobolographic analysis of interactions between fluconazole and essential oils against *Aspergillus fumigatus* showed additive interactions for the combination of fluconazole with lemon, orange and grapefruit oils and an additive interaction with a tendency to synergism for the combination of fluconazole with tangerine oil. Other studies for *Cladosporium cladosporioides* showed additive interactions for the combination of fluconazole with orange and grapefruit essential oil and additive with a tendency for synergism for combination of fluconazole with lemon and tangerine essential oils [26]. Additive interactions between fluconazole and essential oils may be related to the fact that both fluconazole and essential oils inhibit the activity of cytochrome P450 (CYP) enzymes in fungi. Cytochrome enzymes are responsible for the synthesis of ergosterol (the main sterol of the fungal cell membrane). Disturbances in ergosterol synthesis cause changes in the fluidity of the cell membrane, thus inhibiting fungal replication [46, 47]. Animal studies have confirmed that limonene, the main component of citrus essential oils, reduces the activity of CYP2B1 and CYP2B6 [48,49]. While fluconazole inhibits: CYP2C9, CYP2C19, CYP3A4 of cytochrome P450 enzymes [47]. Due to the inhibition of other cytochrome enzymes, the complementary effects of fluconazole and the essential oils appear to elicit additive interactions.

## CONCLUSIONS

The promising results of the conducted *in vitro* tests encourage and justify the need for further research and analysis. This may contribute to the development of new therapeutic methods of fungal infections using known antifungal drugs, the action of which would be supplemented with active substances of natural origin. Searching for natural substances with anti-fungal activity and determining their interactions with known antifungal drugs may turn out to be an interesting trend in research due to the possibility of limiting the use of antibiotics and their side-effects.

The use of the isobolographic analysis method to assess the interaction of a standard drug in combination with an active substance of natural origin in *in vitro* studies on microorganisms is a fairly new procedure. Isobolographic analysis can contribute to introducing natural substances into the therapy of many diseases.

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