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THE POTENTIAL INHIBITORY EFFECT OF *CUMINUM CYMINUM*, *ZIZIPHORA CLINOPODIOIDES* AND *NIGELLA SATIVA* ESSENTIAL OILS ON THE GROWTH OF *ASPERGILLUS FUMIGATUS* AND *ASPERGILLUS FLAVUS*

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ABSTRACT

The goals of this study were to evaluate the effectiveness of *Cuminum cyminum*, *Ziziphora clinopodioides* and *Nigella sativa* essential oils to inhibit the growth of *Aspergillus fumigatus* and *A. flavus* and to evoke ultrastructural changes. The fungi were cultured into RPMI 1640 media in the presence of oils at concentrations of 8, 6, 5, 4, 3, 2, 1.5, 1.25, 1, 0.75 and 0.5 mg/ml in broth microdilution and 2, 1.5, 1 and 0.5 mg/ml in broth macrodilution methods with shaking for 48 h at 28°C. Conidial and mycelial samples exposed to 0.25, 0.5, 1, 1.5 and 2 mg essential oils/ml for 5 days in 2% yeast extract granulated plus 15% Saccharose media were processed for transmission electron microscopy (TEM). Based on broth dilution methods, *C. cyminum* and to a lesser extent *Z. clinopodioides* oils exhibited the strongest activity against *A. fumigatus* and *A. flavus* with MIC_{90} ranging from 0.25 to 1.5 mg/ml, while the oil from *N. sativa* exhibited relatively moderate activity against two above fungi with MIC_{90} ranging from 1.5 to 2 mg/ml. The main changes observed by TEM were in the cell wall, plasma membrane and membranous organelles; in particular, in the nuclei and mitochondria. These modifications in fungal structure were associated with the interference of the essential oils with the enzymes responsible for cell wall synthesis, which disturbed normal growth. Moreover, the essential oils caused high vacuolation of the cytoplasm, detachment of fibrillar layer of cell wall, plasma membrane disruption and disorganization of the nuclear and mitochondrial structures. *Aspergillus fumigatus* and *A. flavus* growth inhibition induced by these oils were found to be well-correlated with subsequent morphological changes of the fungi exposed to different fungistatic concentrations of the oils. Our results show the anti-*Aspergillus* activities of *C. cyminum*, *Z. clinopodioides* and *N. sativa* essential oils, which strengthens the potential use of these substances as anti-mould in the future.

Key words: Antifungal activity, Essential oil, *Cuminum cyminum*, *Ziziphora clinopodioides*, *Nigella sativa* , *Aspergillus*

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INTRODUCTION

Moulds of the genus *Aspergillus* are among the most common fungi in the environment, being found in the air, in the soil, on plants and on decomposing organic matter (22). Because of their powerful hydrolytic enzymes, fungi can cause a high degree of deterioration when present in/on food- and feed-stuffs (23). Thus, the presence and growth of these fungi in food- and feed-stuffs threatens human and animals health. In immunocompromised subjects, inhalation of spores gives rise to aspergillosis, an invasive infection of the lungs or sinuses and its dissemination to other organs (11).

Resistance of *Aspergilli* to some clinically used antifungals brings a worrying clinical prognostic in people attacked by aspergillosis (6, 9). The wide use and sometimes misuse of antimicrobial agents in both human and animal medicine has been responsible for rapid development of resistant strains, toxicity and drug-drug interactions (16, 27). Regarding the increasing clinical importance given to fungi causing infections and the development of drug resistance, many researchers focused on the antifungal properties of plant products.

Plants from Iranian biomes, such as *C. cyminum* (Apiaceae; known as Ziree), *Z. clinopodioides* (Labiatae; known as Avishan) and *N. sativa* (Ranuculaceae; known as black seed) have been used as natural medicines by local populations in the treatment of several diseases (3, 30). Previous studies revealed interesting antimicrobial effects from their essential oils (1, 15, 20). To our knowledge, there is lack of information about their effects on the kinetics of the mycelial growth and germination of *Aspergillus* conidia. The aims of this study were to evaluate the effect of *C. cyminum*, *Z. clinopodioides* and *N. sativa* essential oils on the growth of two important *Aspergillus* species, *A. fumigatus* and *A. flavus*, recognized as potential air- and/or food-borne pathogens.

MATERIALS AND METHODS

Plant materials

The whole aerial parts of *C. cyminum*, *Z. clinopodioides* and *N. sativa* plants, belonging to 3 plant families, were collected from different regions of Khorasan province (northeast of Iran) during 2008. The medicinal plants were selected on the basis of traditional information regarding the treatment of various diseases in Iran. Botanical identification was performed at the Herbarium of Pharmacognosy Department, School of Pharmacy, Mashhad University of Medical sciences, Mashed, Iran.

Extraction of essential oils

Essential oils were isolated by water distillation for 3 h from air-dried materials, using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (8). The oils were stored at −4°C in sealed brown vials until use. The essential oils were assayed at concentrations of 8, 6, 5, 4, 3, 2, 1.5, 1.25, 1, 0.75 and 0.5 in broth microdilution and 2, 1.5, 1 and 0.5 mg/ml in broth macrodilution methods. The stock solutions of oils were prepared according to Souza *et al.* (29). In this study, all general chemical materials were purchased from Merck Company (*Darmstadt, Germany*).

Fungal species and conidia preparation

Aspergillus fumigatus (ATCC 16913) and *A. flavus* (ATCC 16013) strains were used as test microorganisms. In addition, 6 isolates of *A. fumigatus* and *A. flavus*, obtained from air samples, were included in this study. These strains were taken from the Fungal Collection, Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Iran. The fungal strains were precultured on Sabouraud glucose agar slants at 28°C for 10 days. Conidia were taken from the slants

using sterile distilled water containing 0.5% Tween 80. The resulting mixture of conidia and hyphal fragments was vortexed for 15 s and the heavy particles were allowed to settle for 5 min. The resulting suspension was counted in a Neubauer chamber and standardized to concentrations of 1×10^7 conidia/ml. This suspension was further diluted 1:10 with RPMI 1640 broth to final concentrations of 1×10^6 conidia/ml.

Antifungal activity measurements

The MIC_{90} and MFC for 3 essential oils were determined by broth macro- and microdilution methods, according to the protocol in M38-A for filamentous fungi with some modifications (7). For the broth macrodilution method, 900 µl of the final conidia suspensions were mixed with 100 µl of the test essential oil in 12×75 mm test tubes and incubated at 28°C for 48 h. The positive control tube contained 900 µl of conidial suspension plus 100 µl of RPMI 1640, and the negative one contained 1 ml of RPMI 1640 only. The lowest oil concentration inhibiting fungal growth by 90% was identified as the minimal inhibitory concentration (MIC_{90}). In addition, flat-bottom microdilution plates containing 96 wells were employed for the broth microdilution method. One hundred microliters of final conidia suspension were added to each well containing 100 µl of the oil. Positive control was the well containing 100 µl of the inoculum suspension and 100 µl of the RPMI only, and the negative control was a well containing 200 µl of RPMI 1640. The minimum fungicidal concentrations (MFCs) were determined by subculturing 10 µl aliquot from all MIC wells showing no visible growth on to Sabouraud glucose agar plates. Each assay was performed 4 times and the results were expressed as the average of the 4 repetitions.

Transmission electron microscopy (TEM)

Fungal materials obtained from 5-day-old cultures of 2% yeast extracted granulated plus 15% Saccharose media and treated with 0.25, 0.5, 1, 1.5 and 2 mg essential oils/ml were processed for TEM (5). The samples were pre-fixed with 2.5% glutaraldehyde in 0.1M sodium phosphate buffer (PBS), pH 7.2 for 3 h at room temperature, followed by thorough washing with phosphate buffer (3 times, 30 min each). Specimens were then post-fixed for 2 h in 1% aqueous osmium tetroxide (OsO_4) at room temperature and washed in PBS buffer (pH=7.2, 3 times, 15 min each). Samples were dehydrated in a graded acetone series (50%, 70%, 80% and 90%, one time at 20 min for each dilution and 3 times at 30 min in 100% acetone) and embedded in 25% spurr's resin. Blocks were sectioned with a diamond knife (*ultramicrotome Richter OMU3*). Sections about 80 nm thick were collected on gold grids, stained toluidine blue for 2 min and then examined under a Zeiss transmission electron microscope at 80 kV (*120-ILFORD Delta 100 ASA, Zeiss, Germany*).

Statistical analysis

Data were analyzed by the unpaired Student's t test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Inhibition of growth

Results of the inhibitory activities of essential oils on the growth of *A. fumigatus* and *A. flavus* are presented in Table 1. Fungal growth inhibitions were found to be correlated with dose dependent anti-mould activities. Based on broth macrodilution method, *C. cyminum* oil exhibited the strongest activity, with MIC_{90} value of 0.25 mg/ml against tested fungi. The MIC_{90} values for *Z. clinopodioides* were 0.5 mg/ml for *A. fumigatus* and 0.25 mg/ml for *A. flavus* while the oil from *N. sativa* exhibited relatively moderate activity with an MIC_{90} of 1.5 mg/ml for fungi. In broth microdilution method, *C. cyminum* and *Z. clinopodioides* oils showed the MIC_{90} of 1.5 mg/ml for tested fungi while the MIC_{90} values for *N. sativa* oil were 1.5 and 2 mg/ml for *A. fumigatus* and *A. flavus*, respectively. These results generally confirmed those obtained in the broth macrodilution assay.

Fungal development was completely inhibited during 48 h of incubation at concentrations of 2 and 3 mg/ml of essential oils in both macro- and microdilution methods, respectively. Subcultures of these treated inoculums were negative, confirming fungicidal effects against *A. fumigatus* and *A. flavus* at these concentrations.

Table 1. Anti-*Aspergillus* susceptibility of *Cuminum cyminum*, *Ziziphora clinopodioides* and *Nigella sativa* in broth macro- and microdilution methods (mg/ml).

	Broth macrodilution				**Broth microdilution**			
	A. fumigatus		***A. flavus***		***A. fumigatus***		***A. flavus***	
Plant essential oil	**MIC_{90}**	**MFC**	**MIC_{90}**	**MFC**	**MIC_{90}**	**MFC**	**MIC_{90}**	**MFC**
Cuminum cyminum	0.25	0.5	0.25	1	1.5	2	1.5	3
Nigella sativa	1.5	2	1.5	2	1.5	3	2	3
Ziziphora clinopodioides	0.5	1	0.25	0.5	1.5	3	1.5	3

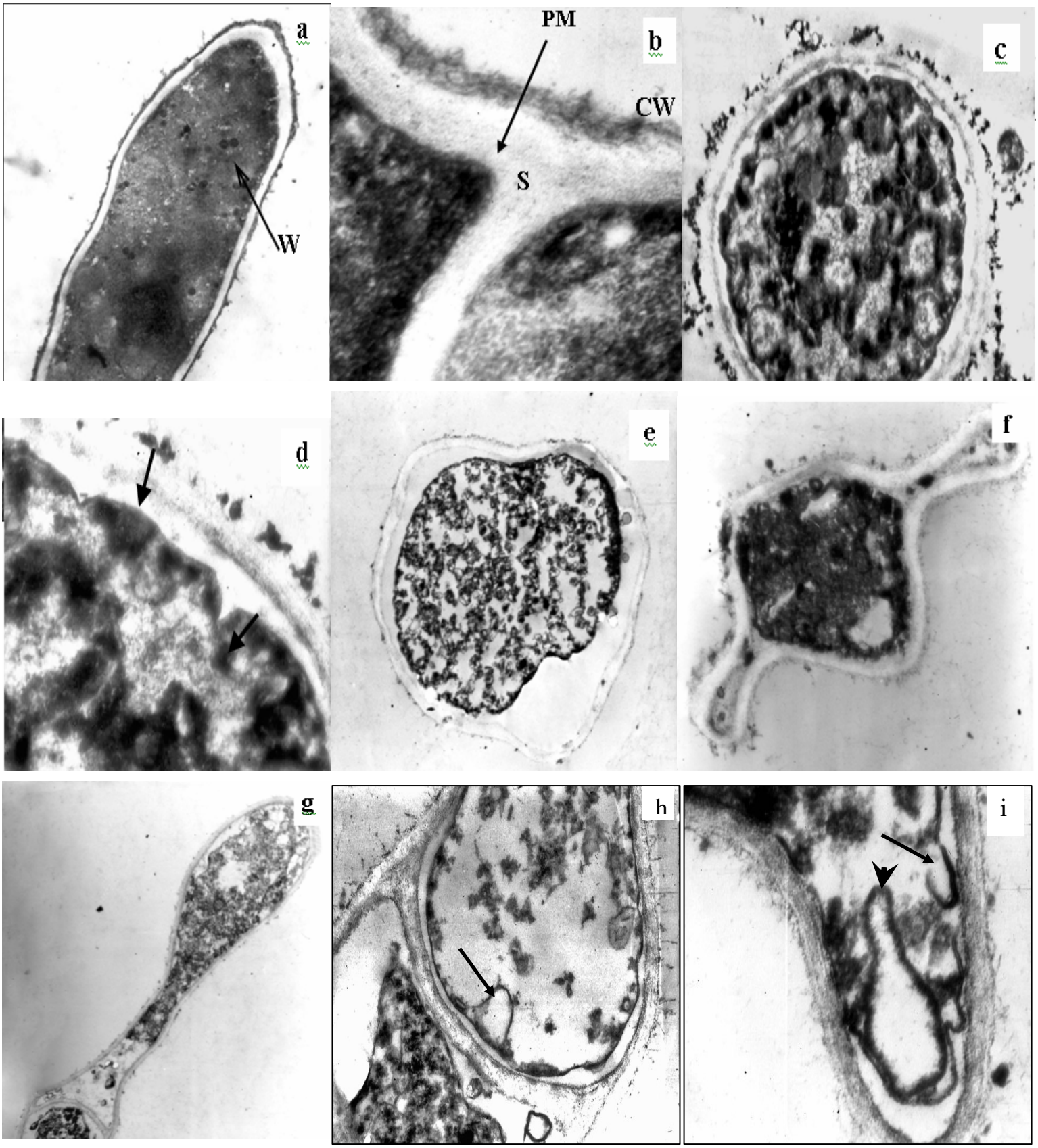
- The values in the table are an average of 4 experiments.

Transmission electron microscopy

In untreated fungi (controls), the cell wall was uniform and thoroughly surrounded by an intact fibrillar layer. Plasma membrane was unfolded with a uniform shape and all the organelles, such as nuclei and mitochondria appeared normal (Fig. 1a and 1b).

TEM observations of *A. fumigatus* and *A. flavus* hyphae and conidia treated at sub-lethal essential oil concentrations were illustrated in Figure 1. After treatment, the normal morphologies of fungal hyphae and conidia were disturbed when the concentration of essential oils increased in the culture media.

The cell shapes were modified and lost their regularity in comparison to the controls. The general alterations of *A. fumigatus* and *A. flavus* hyphae and conidia treated with the oils were similar. The major changes were found on the cell wall, plasma membrane and membranous organelles specially nuclei and mitochondria. The early changes in fungal compartments in the presence of the lowest concentrations of oils (0.25 and 0.5 mg/ml) were noticed in both hyphae and conidia, showing abnormal shaped and swelled hyphae, high vacuolation of the cytoplasm accompanied by vacuole fusion (Fig. 1c and 1d). Subsequent events were loss of normal conidia and hyphae shape, detachment of fibrillar layer of the cell wall (Fig. 1e, 1f and 1g), destruction of hypha memberanous organelles including nuclei and mitochondria, and finally disorganization of cytoplasmic contents accompanied by intensive degradation and lysis of the nucleus and mitochondria (Fig. 1h). The most remarkable changes in fungal compartments were observed in fungi treated with the highest fungistatic concentrations of the oils (1.5-2 mg/ml). Destruction and breaking down of plasma membrane at different sites (Fig. 1i), disorganization of conidial and hyphal cytoplasm and complete lysis of membranous organelles seemed to be lead to cells dead (Fig. 1j and 1k).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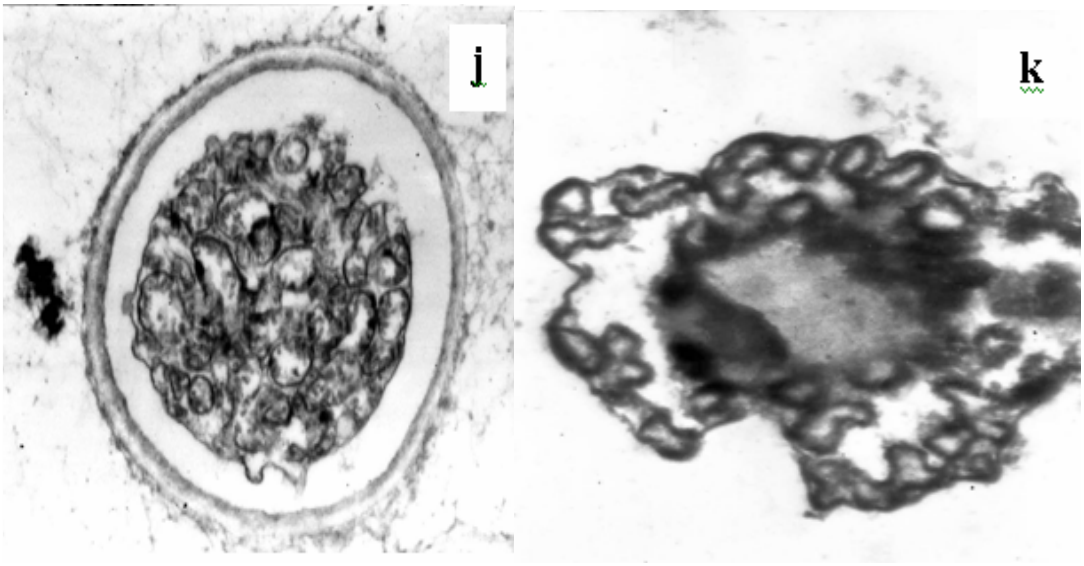

- CW: Cell wall; PM: Plasma membrane; S: septum; W: Woronin body

Figure 1. Transmission electron micrographs of *Aspergillus* species: a) Control conidium of *A. fumigatus* (4400X); b) Control hyphae of *A. flavus* (50000X); c and d) Cross sections of *A. fumigatus* (c, 12000X) and *A. flavus* (d, 30000X) hyphae treated with 2 mg/ml *N. sativa* oil, showing clear separation of plasma membrane from the cell wall, detachment of fibrilar layer of the cell wall, disruption of the cytoplasm and vacuolation of cytoplasm accompanied by vacuole fusion; e and f) Sections of *A. flavus* (e, 7000X) and *A. fumigatus* (f, 12000X) conidia treated with 0.5 mg/ml *C. cyminum* oil, showing increase in vacuolization accompanied by complete detachment of plasma membrane from cell wall (e). Note strong cytoplasmic retraction of conidium (f); g) *A. fumigatus* hyphae treated with 0.25 mg/ml *Z. clinopodioides* oil, showing degradation and discontinuity of plasma membrane (7000X); h) *A. flavus* hyphae treated with 0.25 mg/ml *Z. clinopodioides* oil, showing destruction and lysis of hypha memberanous organelles including nuclei and mitochondria and disorganization of cytoplasmic contents (12000X); i) Arrows indicate destruction and breaking down of plasma membrane with massive formation of membrane-bounded vesicles (50000X); j and k) Collapsed conidia treated with 0.25 mg/ml of *C. Cyminum* oil in *A. fumigatus* (j, 7000X) and *A. flavus* (k, 12000X), which finally resulted in cells dead.

DISCUSSION

This study described the effectiveness of *C. cyminum*, *Z. clinopodioides* and *N. sativa* essential oils against *Aspergillus* species by both macro- and microdilution assays and ultrastructural changes of the fungal species tested. In broth macrodilution test, the oil concentrations of *C. cyminum* at 0.25 mg/ml, *Z. clinopodioides* at 0.5 and 0.25 mg/ml and *N. sativa* at 1.5 mg/ml showed fungistatic activity against *A. fumigatus* and *A. flavus*, respectively, while this effect was observed with values of 1.5 mg/ml for *C. cyminum* and *Z. clinopodioides*, and 1.5 and 2 mg/ml for *N. sativa* oils in broth microdilution method, with 90% growth inhibition after 48 h of incubation. The MICs in broth macrodilution cultures were different from the MICs for cultures in broth microdilution method, indicating no significant difference between both tests. In several studies, the microdilution MICs demonstrated interlaboratory agreement with most of the drugs with the values higher than

or similar to the macrodilution MICs and discrepancies between the two tests were not statistically significant (13,14). The different values for MIC_{90} obtained with the oils incorporated in broth macro- or microdilution tests showed that the level of antifungal activity of essential oils was closely dependent on the screening method used and fungi tested, as previously reported (10).

This study demonstrated that *C. cyminum* oil had the highest inhibitory effect against *A. fumigatus* and *A. flavus* among the oils tested. The different activity of these oils may be due to their different components, the structural configuration of the constituent components and their functional groups and possible synergistic interactions between components (12). The main constituents of *C. cyminum* oil were pinene, cineole and linalool, while the main components found in Z. clinopodioides oil were pulegone, 1,8-cineole and limonene, and in *N. sativa* oil the components were *trans*-anethole and *p*-cymene (4,21,24,25). Aligiannis *et al.* (2) proposed a classification for plant materials, based on MIC results in broth macrodilution test as follows: strong inhibitors (MIC up to 0.5 mg/ml); moderate inhibitors (MIC between 0.6 and 1.5 mg/ml); weak inhibitors (MIC above 1.6 mg/ml). According to Table 1, a strong activity against *Aspergillus* species was indicated for the oils from *C. cyminum* and Z. *clinopodioides*. The oil from *N. sativa* presented moderate to weak activity. In respect to the oils activity, no significant difference was observed between the *Aspergillus* species tested. The MIC results for *Aspergillus* species in our study were those found by Naeini *et al*. (20) for *Candida albicans*.

The TEM of essential oils-treated fungi in comparison with untreated samples clearly showed dose-dependent changes of fungal cells, especially on membranous structures. Interestingly, growth inhibitions of *A. fumigatus* and *A. flavus* were found to be well correlated with correspondence morphological changes of the fungi exposed to different fungistatic concentrations of the oils. As concerned the cell wall, it was observed that the surfaces of the hyphae and conidia treated by oils became rough in contrast to the control group. As the concentration of oils increased, the cell gradually became smaller, the cell wall was disrupted and became rough and villiform. Subsequently, the cell wall became very thin and even seemed to disappear in some old hyphae. In a study conducted by Ghfir (18), a deformation of the apices of *A. fumigatus* growing hyphae was observed in the presence of *Hyssopus officinalis* essential oil. In another study, cell wall degradation was also observed in *C. albicans* cells treated with *Carica papaya* latex sap (17). Such modifications induced by essential oils may be related to the interference of essential oil components with enzymatic reactions involved in cell wall synthesis, thus affecting fungal morphogenesis and growth; however that remains to be proved.

The oils tested, besides the increase in vacuolization, showed a strong alteration in the cytoplasmic membrane. The plasma membrane of *Aspergillus* species was seen to be irregular and dissociated from the cell wall and cut into small fragments; these membrane segments were dispersed into the cytoplasm. These changes were usually found in fungi treated with imidazole components (26).

Finally, the membranous organelles were disrupted following treatment with oils. The membrane-disruptive activity of essential oil components may be closely associated with the interference with enzymatic reactions of the membrane, such as respiratory electron transport, proton transport, and coupled phosphorylation steps (19). Moreover, a marked depletion of cytoplasmic contents of hyphae accompanied by lysis and disruption of membranes of major organelles, such as nuclei and mitochondria indicated that in high fungistatic concentrations the oils passed not only through the cell wall but also through the plasma membrane and then interacted with membranous structures of the cytoplasmic organelles. The present results clearly showed that the oils were able to inhibit fungal growth by changing the cell uniformity via direct interaction with either cell wall or cytoplasmic membranes. From the observations of TEM, no

significant morphological changes were found in the hyphae and conidia exposed to the low concentration of the oils, but the conidia exposed to relatively higher concentrations of oils collapsed. There were minor differences in morphological changes of different fungi based on the oils tested. It is considered that this phenomenon is related to constituents of oils that possess a polar functional group like hydroxyl group or have a charged group such as anionic group or cationic group (28).

From the results of this study, it might be concluded that *C. cyminum*, *Z. clinopodioides* and *N. sativa* oils possess antifungal activities to inhibit the growth of *A. fumigatus* and *A. flavus*. The antifungal activity of the oils was evident at the morphological level. Due to the antifungal activity of these oils and their availability as natural volatile products, they might be of use in future studies of antifungal agents.

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