In vitro Inhibitory Action of the Essential Oils of *Origanum Vulgare* and *Rosmarinus Officinalis* against *Aspergillus Fumigatus*

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ABSTRACT

Aspergillus fumigatus is the main etiological agent of aspergillosis. Considering azole antifungal drug resistance in *A. fumigatus*, which compromises treatment, new alternatives are needed. Among them, essential oils (EOs) can be an alternative treatment, having shown positive results in inhibiting phytopathogenic fungi *in vitro*. We aimed to determine the *in vitro* antifungal activity of *Origanum vulgare* L. subsp. *hirtum* (Link) (oregano) and *Rosmarinus officinalis* L. (rosemary) EOs alone and in association (*O. vulgare* + *R. officinalis*) against *A. fumigatus*. EOs were analyzed by gas chromatography (GC-FID and GC/MS systems), and analyses showed that the major components of *O. vulgare* EO were carvacrol (67.8 %), p-cymene (14.8 %), and thymol (3.9 %); for *R. officinalis,* they were the monoterpenes 1,8-cineole (49.1 %), camphor (18.1 %) and α-pinene (8.1). For biological assays, five EO concentrations, 0.2; 0.4; 0.6; 0.8 and 1.0 %, were used in disk diffusion and agar dilution tests for 21 days. In disk diffusion, *O. vulgare* EO alone and in association (*O. vulgare* + *R. officinalis*) showed fungicidal activity at all concentrations. In agar dilution, inhibitory action was demonstrated from 0.6 % for *O. vulgare* EO and in association (*O. vulgare* + *R. officinalis*). *R. officinalis* EO at 1.0 % showed no fungal growth, determining the minimum inhibitory concentration (MIC). The present study demonstrated inhibitory actions of *O. vulgare* and *R. officinalis* EOs in *A. fumigatus*. GC analyses corroborated the literature regarding their antibacterial and antifungal effects. However, further *in vitro* and *in vivo* studies are needed to evaluate EOs as alternative antifungals for treating aspergillosis.

ABBREVIATIONS

EO essential oil
MGI mycelial growth index
MIC minimum inhibitory concentratione

* ACMM, ADD and FMN contributed equally to this work.

Introduction

Aspergillus fumigatus is a fungus of the Trichocomaceae family and the main etiological agent of aspergillosis, which promotes a series of diseases, from allergic syndromes to invasive aspergillosis, affecting immunocompetent patients, and especially those who are immunocompromised [1, 2].

Azole antifungals are used in the prophylaxis and pharmacological treatment of aspergillosis, acting on the cytochrome P450 enzymes of the fungus and inhibiting the 14α-demethylation of lanosterol and the biosynthesis of ergosterol, an essential component of the fungal cell membrane [1, 3]. However, therapy with this therapeutic class is linked to toxicity, high cost, drug interactions, and difficulties in assessing the therapeutic response [4].

There are reports in the literature that *A. fumigatus* can adapt to the presence of azoles by spontaneous mutation, which facilitates the resistance of the species, compromising the treatment of patients [1–4]. Thus, it is necessary to seek other therapeutic methods with broad-spectrum inhibitory activities [4].

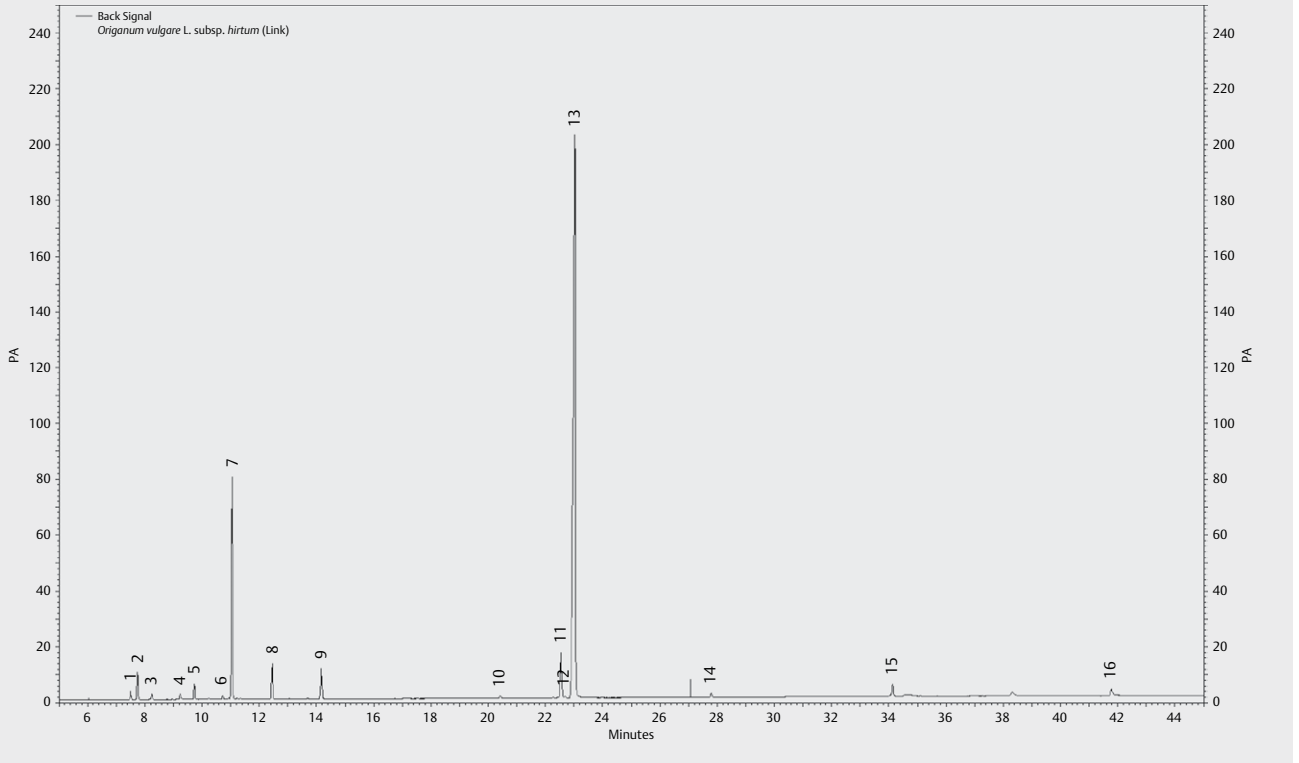
Essential oils (EOs) can be used as an alternative in the treatment of aspergillosis, as they have shown positive results in the *in vitro* inhibition of phytopathogenic fungi [4]. They are volatile, transparent, or slightly yellowish liquids, which dissolve in lipids and organic solvents and contain secondary metabolites capable of inhibiting or hindering microbial growth, exerting action on the microorganisms' membrane and cytoplasm [5].

Among the EOs that have shown significant *in vitro* effects against the fungus *A. fumigatus* are *Origanum vulgare* L. (oregano) and *Rosmarinus officinalis* L. (rosemary), both from the Lamiaceae family. These EOs are mainly composed of terpenoids which are related to the antimicrobial and antioxidant activities of these species [4–6]. The association of these EOs in subinhibitory concentrations can exert synergistic effects on the *Aspergillus* genus, making them more effective than isolated application, due to the association of major metabolites [7].

Considering the azole antifungal drug resistance in *Aspergillus fumigatus*[1–4], new alternatives for the treatment of aspergillosis are needed. Therefore, this work aimed to determine the *in vitro* antifungal activity of the EOs of *Origanum vulgare* L. subsp. *hirtum* (Link) and *Rosmarinus officinalis* L., alone and in association (*O. vulgare* + *R. officinalis*), on *Aspergillus fumigatus*.

Results and Discussion

GC analyses of the EOs from *O. vulgare* showed that the major components were carvacrol (67.8 %), p-cymene (14.8 %), thymol (3.9 %), γ-terpinene (2.5 %), β-linalool (2.2 %), and α-pinene (1.6 %) (▶ **Fig. 1**; ▶ **Table 1**); whereas in *R. officinalis* they were 1,8-cineole (eucalyptol; 49.1 %), camphor (18.1 %), α-pinene (8.1 %), 1-terpineol (4.6 %), α-terpineol (3.1 %), camphene (3.1 %), β-pinene (2.7 %), p-cymene (1.9 %), and limonene (1.6 %), as well as β-linalool (1.2 %) and terpinen-4-ol (1.0 %), among others (▶ **Fig. 2**; ▶ **Table 2**).e



▶ **Fig. 1** Chromatogram of the essential oil of *Origanum vulgare* L. subsp. *hirtum* obtained by gas chromatography coupled with mass spectrometry and flame ionization detector (GC-FID and GC/MS systems). Analysis methods: GC/FID: Gas chromatograph Agilent 7820 A. Column: RXI-5MS 30 m x 0.25 mm × 0.25 µm (Restek). Temp.: Column: 50 °C (0 min), 3 °C/min at 200 °C. Injector: 220 °C Split: 1/50. FID detector: 220 °C. Injection volume: 1 µl. Sample prepared in 2 % w/v ethyl acetate. GC/MS: GC/MS-QP2010 chromatograph operating at the same conditions.

The *Origanum* genus is characterized by a wide range of volatile secondary metabolites, which are widely used in food, pharmaceutical and cosmetic industries, mainly in perfumery because of their spicy fragrance. They are also used as a culinary herb, flavoring beverages and food products, and as a food preservative [8]. The major components in *O. vulgare* EO, the monoterpenic phenols carvacrol and thymol, and the monoterpene hydrocarbon γ-terpinene, have been reported for their antibacterial and antifungal effects [9, 10], corroborating our findings of its fungicidal activity at all tested concentrations in the disk diffusion method. Furthermore, p-cymene, detected in both EOs but with higher concentration in the *O. vulgare* EO, has shown a range of biological activity including antioxidant, anti-inflammatory, antinociceptive, anxiolytic, anticancer, and antimicrobial effects [11].

For *R. officinalis*, among the volatile monoterpenes, the major components of the EO, 1,8-cineole (eucalyptol) and camphor, have been reported as exhibiting strong growth-inhibitory effects on plants and are considered to be involved in plant competition [12]. Moreover, 1,8-cineole possesses *in vitro* antimicrobial and fungicidal activities [13, 14], which could enhance the antimicrobial effects of the other antiseptics [14]. The antimicrobial activities of the isomers and enantiomers of pinene, also among the major components of the analyzed *R. officinalis* EO, were tested, showing microbicidal activity against bacterial and fungal cells [15, 16]. Likewise, terpinen-4-ol showed strong *in vitro* fungicidal activity [13]. In fact, rosemary oil has been reported to have antibacterial, antifungal, and antioxidant properties. It is used as a food seasoning, and also demonstrates a number of applications in managing or curing inflammatory diseases and diabetes mellitus, among others [17].

Antifungal susceptibility tests are performed on pathogenic fungi, especially when infections are invasive, relapsing, or therapy fails, when they have inherent or acquired resistance, or when susceptibility cannot reliably be predicted from the species identification alone. Antifungal susceptibility testing (AFST) is also important in resistance surveillance, epidemiological studies, and for comparison of the *in vitro* activity of new and existing agents [18, 19].

In the disc-diffusion method performed here, the *O. vulgare* EO alone showed fungicidal action on *A. fumigatus*, which presented susceptibility at all concentrations, with no growth of the fungus for 21 days; results were compared with the positive control under the same conditions as the samples. On the other hand, for strains treated with the *R. officinalis* EO at 0.2; 0.4; 0.6; 0.8 and 1.0 %, no inhibition halos were observed in any of the tested concentrations, with gradual growth of fungal colonies (▶ **Table 3**).

O. vulgare is known for its interesting antimicrobial activity and is even considered an alternative antimicrobial for use in food preservation systems. Carmo et al. (2008) evaluated the effectiveness of *O. vulgare* EO in inhibiting the growth of 12 strains of *Aspergillus* species, showing dose-dependent antifungal activity. They observed that at concentrations of 80 and 40 µL/mL there was a fungicidal effect on the species *A. flavus*, *A. fumigatus* and *A. niger*, providing total inhibition of mycelial growth for 14 days. The strains treated with *O. vulgare* EO were compared with ketoconazole, and this drug did not show significant inhibition, exhibiting progressive mycelial growth [20].

Considering our results and the azole antifungal drug resistance in *A. fumigatus* [1–4, 21], *O. vulgare* EO could be a future alternative in the treatment of aspergillosis after further *in vitro* and *in vivo* studies.

Regarding *R. officinalis* EO, *in vitro* antifungal effects on *Aspergillus* species have also been tested [22–24]. Bomfim et al. (2019) analyzed the antifungal activity of the *R. officinalis* EO on *A. flavus*, using scanning electron microscopy, and observed a reduction in the size of the conidiophores and the thickness of the hyphae in the concentration at 250 µg/mL; however, in the sample treated with 500–1000 µg/mL of *R. officinalis* EO, there were no conidiophores. When evaluating the action of this EO on ergosterol, the main component of the fungal cell membrane, total inhibition was seen at the concentration of 500 µg/mL Similarly, Baghloul et al. (2016) tested the antifungal effect of *R. officinalis* EO against the fungal strain *A. niger* and observed that this EO showed activity on all strains tested with a minimum inhibitory concentration (MIC) of 0.5 % [22]. In contrast, in the work carried out by Fu et al. (2007), this EO exhibited less inhibitory activity in *A. niger* (MIC of 1.0 %), compared to *Candida albicans* (MIC of 0.250 %) [23].

Therefore, thinking about our findings and these studies, the reports that demonstrate that the major components of *R. officinalis* EO could enhance the antimicrobial effects of the other antiseptics [13–15] corroborate our results of the EOs' association. *R. officinalis* and *O. vulgare* EOs in combination have been reported as controlling postharvest pathogen *Aspergillus* species and autoch-e

▶ **Table 1** Chemical composition of volatiles in *Origanum vulgare* L. subsp. *hirtum* essential oil by gas chromatography coupled with mass spectrometry and flame ionization detector (GC-FID and GC/MS systems).

Peak	RI_{calc}	Constituents ID	Content (%)	Identification Method
1	916	α-thujene	0.5	RI, MS
2	922	α-pinene	1.6	RI, MS
3	934	camphene	0.5	RI, MS
4	959	β-pinene	0.6	RI, MS
5	972	β- myrcene	1.1	RI, MS
6	996	α-terpinene	0.2	RI, MS
7	1004	p-cymene	14.8	RI, MS
8	1039	γ-terpinene	2.5	RI, MS
9	1082	β-linalool	2.2	RI, MS
10	1237	methylthymol	0.2	RI, MS
11	1290	thymol	3.9	RI, MS
12	1293	p-cymen-7-ol	0.2	RI, MS
13	1302	carvacrol	67.8	RI, MS
14	1420	β-caryophyllene	0.3	RI, MS
15	1578	β-caryophyllene oxide	1.3	RI, MS
16	1768	(Z)-7-hexadecenal	1.3	RI, MS
		others	1.1	RI, MS

MS = Mass Spectrometry; RI = Retention Index. RI obtained using an RXI-5MS column. OBS.: Peaks less than 0.1 % were excluded. The linear RI were determined from the C12–C17 hydrocarbon mixture, purchased from Sigma Aldrich, reference 48244, analyzed under the same conditions as the essential oil sample.

thonous mycoflora of vegetables [7], also supporting our results. The data presented in ▶ **Table 4** indicate that the association of *O. vulgare* and *R. officinalis* EOs showed inhibitory activity on *A. fumigatus* at all concentrations evaluated during the 21 days of monitoring, thus demonstrating the antifungal effect resulting from the combined application of these EOs, while the positive control had a diameter < 10 mm (without inhibitory activity).

Alexa and co-workers (2018) observed the *in vitro* inhibitory action of EOs of two species of the Lamiaceae family, *Thymus vulgaris* and *Salvia officinalis*, alone and in association, on the fungus *Fusarium graminearum*. In this study, when the EOs were associated with a concentration of 0.06 % (v/v), they showed higher antifungal activity than when applied alone and significant differences between the concentrations when compared with the positive control, indicating synergistic antifungal, allelopathic, and anti-proliferative potential [25]. This synergistic antifungal effect corroborates our results with *R. officinalis* and *O. vulgare* EOs in combination.

There are reports that some compounds present in the chemical composition of *O. vulgare* and *R. officinalis* EOs, such as carvacrol, thymol, and terpenes, are associated with antimicrobial activities, affecting the growth and morphogenesis of microorganisms, increasing the permeability of the cytoplasmic membrane, and considerably decreasing cytoplasmic adenosine triphosphate (ATP), destabilizing the membrane [9, 17, 26–29].

Nonetheless, the effectiveness of the EOs of *O. vulgare* and *R. officinalis* in combination against *A. fumigatus* could also be related to the combined action of the different minority components present in the volatile fraction, and to the increase in their concentrations when the two EOs are associated. Thus, although carvacrol and thymol present in oregano show antibacterial and antifungal effects [9], γ-terpinene present in *O. vulgare* EO has also showed fungicidal activity, with minimum fungicidal concentration (MFC) values between 125 and 500 µg/mL [28]. Likewise, terpenes, present in both EOs, can disorganize the cell membrane, and therefore promote its lysis [17]. Furthermore, oxygenated monoterpene-rich volatile oils, such as that of *R. officinalis*, have been reported as potential antifungal agents for dermatophytes, besides being related to broad-spectrum antibacterial activities [30]. So, results obtained with agar dilution method were particularly important for determining the MIC. Moreover, the disk-diffusion method is reportedly not accurate in detecting resistance mechanisms and not fully elucidated for certain microorganisms, while the agar dilution method, albeit more laborious and extensive, is standardized and can be a reference to evaluate new drugs [31].

Interestingly, using the agar dilution method (▶ **Table 5**), *R. officinalis* EO did not show fungistatic activity at concentrations of 0.2; 0.4; 0.6 and 0.8 %, with mycelial growth since the first evaluation. However, at 1.0 % there was no fungal growth (MIC). The nonparametric repeated measures ANOVA (Friedman Test) showed significant differences among concentrations and over time for both *O. vulgare* and *R. officinalis* EOs ($p < 0.001$). For *O. vulgare*, significant differences among concentrations were detected by pairwise comparisons (Durbin-Conover) for the 0.2 and 0.4 % compared to 0.6, 0.8 and 1 % ($p < 0.001$), while over time, these differences were not detected only between the first and the seventh days ($p = 0.004$ for 1st and 7th days compared to 14th day; $p < 0.001$ for all days compared to 21st day). For *R. officinalis*, all lower concentrations significantly differed from the 1 % (MIC), and there were significant differences in almost all compared times ($p < 0.001$ for all pairwise comparisons), except when comparing the 14th day to the 21st day. Fu et al. (2007) also demonstrated that *R. officinalis* EO showed an inhibitory effect on the *Aspergillus* genus at a concentration of 1 %, but further studies based on this concentration are required [23].

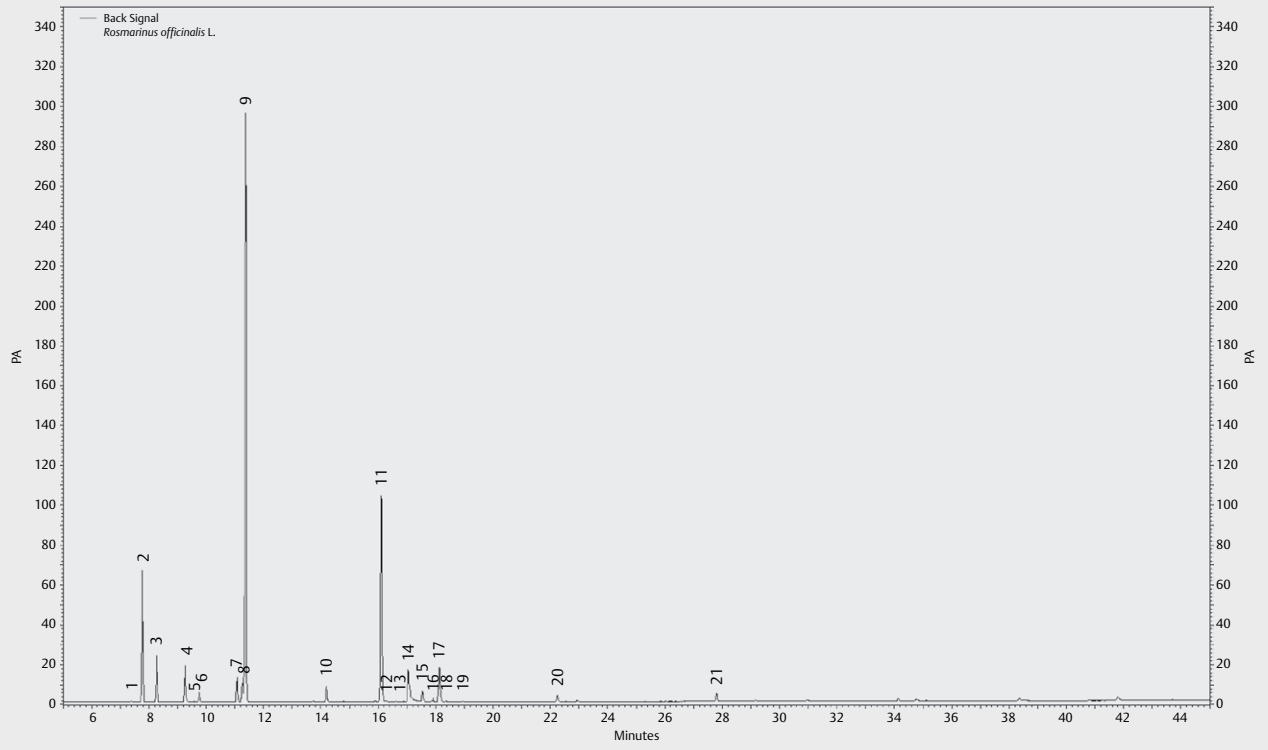
The MGI of *A. fumigatus* treated with the EOs alone was also analyzed in the interval of 7 days, for 3 weeks. *O. vulgare* EO showed total inhibitory action on the fungus from the concentration of 0.6 % upwards (▶ **Table 5**), while the growth in diameter of the colonies, at 0.2 and 0.4 %, was belated, being observed from the 14th and 21st days, respectively. For *R. officinalis* EO, progressive development of the fungal colony was observed on the 1st day of monitoring. After the 7th day, the speed of the MGI stabilized between concentrations (▶ **Table 5**).

O. vulgare EO, as expected, provided a significant reduction in the fungal colony development, when compared to *R. officinalis* EO alone ($p < 0.001$). Some authors have reported that *O. vulgare* EO showed antifungal activity, obtaining different degrees of mycelial inhibition, and reinforcing its promising use in the treatment of aspergillosis [4, 32]. Although the most common secondary metabolites ranged between carvacrol and thymol [5], or terpinen-4-ol, thymol and cis-sabinene hydrate [4, 33], the last of these was not identified in our sample, and it should be recalled that the chemical composition of a plant species may vary with the geographical origin and the harvest period [5], since environmental factors may influence types and contents of bioactive substances [34, 35]. In addition, according to the widely accepted taxonomy, six subspecies of *O. vulgare L.* have been recognized [36], and the botanical subspecies which we evaluated (*O. vulgare* L. subsp. *hirtum*) does not have high levels of this component; indeed, our findings are in accordance with the typical composition of this subspecies (high levels of carvacrol and p-cymene) [37–39].

Regarding *R. officinalis* EO, the major volatile constituents found in our study, the monoterpenes 1,8-cineole, camphor and α-pinene, are possibly related to its antifungal activities [6], even though only at 1.0 %. Our results corroborate the literature, where Fu et al. (2007) also showed that *R. officinalis* EO had an inhibitory effect on *A. niger* of 1 % [23].

Analysis of the association of the EOs showed MIC values of 0.6, 0.8 and 1.0 %, and slow growth of the fungal colony of *A. fumigatus* at 0.2 and 0.4 % (▶ **Table 6**), where the non-parametric repeated measures ANOVA (Friedman Test) also showed significant differences among concentrations and over time ($p < 0.001$). Supporting this, the pairwise comparisons (Durbin-Conover) showed no significant differences only at 0.6–0.8 %; 0.6–1 % and 0.8–1 % ($p < 0.001$ for the other comparisons), while over time, significant differences were not detected only between the first and the seventh days ($p = 0.004$ for 1st and 7th days compared to 14th day; $p < 0.001$ for all days compared to 21st day). These results corroborated MGI, which showed prolonged growth on the 14th and 21st days at 0.2 and 0.4 %, respectively (▶ **Table 6**).

As expected, significant differences were also found in the colony development of *A. fumigatus*, when the EO of *R. officinalis* alone was compared with the combined EOs ($p < 0.001$), but not when the EO of *O. vulgare* alone was compared with the association of EOs ($p = 0.871$).



▶ **Fig. 2** Chromatogram of the essential oil of *Rosmarinus officinalis* obtained by gas chromatography coupled with mass spectrometry and flame ionization detector (GC-FID and GC/MS systems). Analysis methods: GC/FID: Gas chromatograph Agilent 7820 A. Column: RXI-5MS 30 m × 0.25 mm × 0.25 µm (Restek). Temp.: Column: 50 °C (0 min), 3 °C/min at 200 °C. Injector: 220 °C Split: 1/50. FID detector: 220 °C. Injection volume: 1 µl. Sample prepared in 2 % w/v ethyl acetate. GC/MS: GC/MS-QP2010 chromatograph operating at the same conditions.

The similar results between *O. vulgare* EO alone or in association suggested the presence of time-dependent fungistatic action in the 1st week at 0.2 % and in the 2nd week at 0.4 %. However, from this period on, a decline was seen in the inhibitory action, possibly due to the time of exposure of *A. fumigatus* to concentrations, and consequent fungal development, although this causality relationship was not statistically verified. Other studies have corroborated this trend, with fungi and bacteria, even using bioactive compounds like carvacrol or an association between EOs [7, 29].

In a study that conducted time series transcriptome analyses on three clinical *A. fumigatus* isolates after the addition of sub-lethal concentrations of itraconazole, Hokken et al. (2019) verified that this species adapts to the presence of an antifungal compound by the immediate up-regulation of several drug-efflux membrane transporters, activation of signaling cascades, and various alterations in gene expression in the ergosterol biosynthesis and phospholipid biosynthetic processes. Therefore, even if there is no mutation involved in the resistance process, the fungus can reveal rapid phenotypic plasticity [3]. As such, the prevalence of azole resistance in *A. fumigatus* emphasizes the importance of other therapeutic strategies with broad-spectrum inhibitory activities, such as EOs, which cause the loss of the integrity and rigidity of the fungal cell wall, reducing or inhibiting the number of conidia [24].

Combinations of individual oils may lead to additive, synergistic, or antagonistic effects, which raise industrial interest in naturally produced medical products and food preservatives [23]. Although the mechanism of antimicrobial activity when there is an association of EOs is not fully understood, and further studies are needed to assess the inhibitory effects [23], our report provides a basis for further exploration and use of these volatile oils in human health and food safety.

In conclusion, the present study demonstrated inhibitory actions of *O. vulgare* and *R. officinalis* EOs on *A. fumigatus*. In the disk diffusion, no visible growth of the fungus was observed with *O. vulgare* EO and EOs in association. Moreover, in the agar dilution test, these EOs showed fungicidal activity from 0.6 %, and fungistatic activity at 0.2 and 0.4 % in the 1st and 2nd week, thus demonstrating a time-dependent effect, while *R. officinalis* EO alone exhibited fungicidal action at 1 % (MIC). However, further studies are needed to evaluate these EOs and their major metabolites as alternative antifungals in treating aspergillosis.

Materials and Methods

The clinical isolate of *A. fumigatus* strain was supplied by Exame Laboratory, of the Diagnostics of America S.A (DASA) group. As the phenotypic identification of fungal colonies may be inaccurate due to taxonomic changes, requiring alternative methods to confirm the species [40], VITEK MS equipment (BioMérieux, France) and microscopic observation were also used here. The VITEK MS is a mass spectrometry system, which consists of laser ionization/desorption assisted by a matrix (MALDI-TOF - Matrix Assisted Laser Desorption Ionization-Time of

▶ **Table 2** Chemical composition of volatiles in *Rosmarinus officinalis* L. essential oil by gas chromatography coupled with mass spectrometry and flame ionization detector (GC-FID and GC/MS systems).

Peak	RI_{calc}	Constituents ID	Content (%)	Identification Method
1	913	α-thujene	0.1	RI
2	923	α-pinene	8.1	RI, MS
3	935	camphene	3.1	RI, MS
4	960	β-pinene	2.7	RI, MS
5	968	5-methyl-3-heptanone	0.1	RI, MS
6	972	β- myrcene	0.7	RI, MS
7	1005	p-cymene	1.9	RI, MS
8	1009	limonene	1.6	RI, MS
9	1012	1,8-cineole (eucalyptol)	49.1	RI, MS
10	1082	β-linalool	1.2	RI, MS
11	1130	camphor	18.1	RI, MS
12	1134	citronellal	0.2	RI, MS
13	1143	isopulegol	0.1	RI, MS
14	1153	1-terpineol	4.6	RI, MS
15	1166	terpinen-4-ol	1.0	RI, MS
16	1174	isoborneol	0.2	RI, MS
17	1180	α-terpineol	3.1	RI, MS
18	1186	cis-dihydrocarveol	0.1	RI, MS
19	1201	trans-dihydrocarveol	0.1	RI, MS
20	1283	bornyl acetate	0.6	RI, MS
21	1421	β-caryophyllene	0.7	RI, MS
		others	2.7	

MS = Mass Spectrometry; RI = Retention Index. RI obtained using an RXI-5MS column. OBS.: Peaks less than 0.1 % were excluded. The linear RI were determined from the C12–C17 hydrocarbon mixture, purchased from Sigma Aldrich, reference 48 244, analyzed under the same conditions as the essential oil sample.

Flight) of chemical compounds, to ionize a sample for the gas phase [41, 42]. Microscopic observation is used to identify filamentous fungi based on their morphological characteristics [43]. For this, after the incubation time, with the aid of a sterile swab, isolates of *A. fumigatus* were inoculated in a 2 mL Eppendorf tube (Sigma Aldrich, SP/Brazil) containing 900 µL of 70 % ethanol. The suspension was vortexed and centrifuged at 10 000 rpm [~13957 X g (RCF)] for 2 minutes, using a NT800 microcentrifuge (NovaTecnica, SP/Brazil). The supernatant was discarded with the aid of a pipette and, subsequently, 40 µL of 70 % formic acid was added, until the new suspension was complete. Then, 40 µL of acetonitrile was added; the suspension was vortexed and centrifuged at 10 000 rpm for 2 minutes. One µL of the supernatant was inoculated on a slide (spots) and, having awaited its total drying, was taken for analysis on the VITEK MS equipment for identification of the species [44]. For microscopic observation, lactophenol-cotton blue stain was used to differentiate the genus and species. The fungal colony was picked out with the aid of a sterile disposable loop, depositing it on a sterile slide. Two drops of the lactophenol-cotton blue were then added, and the coverslip was placed over the fungal sample. Colony characteristics were analyzed using a 40x objective microscope, evaluating the structures of hyphae, conidia, and phialides [43].

After identification, the clinical isolate of *A. fumigatus* strain was then kept on Sabouraud dextrose agar at a temperature of 28 ± 2 °C. All tests performed to assess the inhibitory activity were executed in triplicate.

The EOs of *Origanum vulgare* L. subsp. *hirtum* (Link) (origin: Turkey; batch: 1613) and *Rosmarinus officinalis* L. (origin: Brazil; batch: 1612) were obtained from the Laszlo company (Belo Horizonte, MG/Brazil). The herb of both species was evaluated for the content and composition of essential oil by steam-distillation followed by GC-MS and GC-FID (gas chromatography coupled with mass spectrometry and flame ionization detector).

Chromatographic analyses of both EOs were performed under the same conditions, on an Agilent 7820 A chromatograph (Agilent Technologies Brazil Ltda, Belo Horizonte, MG/Brazil) coupled to the Flame Ionization Detector (GC/FID) and Shimadzu GC/MS-QP2010 Ultra chromatograph (Shimadzu of Brazil, SP/Brazil) equipment. In the Agilent 7820 A chromatograph, both the injector and the detector were operated at 220 °C . The column oven was operated with the following temperature setting: 50 ° C (0 min), 3 ° C/min at 200 °C. The injector was operated in split mode with a 1/50 split ratio, and the carrier gas used was hydrogen, at a flow of 2.98 mL/min. The column used for the separation of the components was the RXI-5MS [length 30 meters, internal diameter 0.25 mm and film thickness 0.25 µm (Restek)]. The linear retention index (RI) was calculated using a hydrocarbon mixture, C12-C17, purchased from Sigma Aldrich (SP/Brazil), reference 48244. In the Shimadzu GC/MS-QP2010 chromatograph, the operating conditions of the injector and detector and the oven temperature programming were the same as previously described. The carrier gas used was helium, at an operating flow of 0.98 mL/min. The column used for separation was the NST05 (30 m × 0.25 mm × 0.25 µm). The ion source and the interface were operated at a temperature of 220 °C.

For the susceptibility tests by disc diffusion and agar dilution, the EOs of *O. vulgare* L. and *R. officinalis* L., isolated and in combination (*O. vulgare* + *R. officinalis*), were analyzed at concentrations 0.2; 0.4; 0.6; 0.8 and 1.0 % (v/v).

The active ingredient of 22 % itraconazole (Fagron) was obtained commercially from a pharmacy in the Federal District and used as positive control at 100 µg/mL [45, 46]. The choice of antifungal agent and the respective dilutions with dimethyl sulfoxide (DMSO) for positive control followed the recommendations of M38-A [45, 46], with some changes. During the pilot test phase, the *Aspergillus fumigatus* strain used was not sensitive to the usual concentrations of itraconazole (from 0.03 to 16 µg/mL) [45]. Thus, higher concentrations of itraconazole were previously tested: 50 µg/mL, 70 µg/ mL, 80 µg/mL and 100 µg/mL; the latter was the one that expressed the best inhibitory response and was thus chosen for positive control purposes.

For the negative control, plates free of antifungal agents were used, and 1 mL of autoclaved distilled water was applied per unit of the triplicate, performed to replace the solutions of the EOs [7, 47].

▶ **Table 3** Results of the disk-diffusion test, according to the diameter of the inhibition halos of *Origanum vulgare* L. subsp. *hirtum*, *Rosmarinus officinalis* L. essential oils and reference drug on *Aspergillus fumigatus*, for 21 days.

Antifungal	Colony development assessment in days (diameter in mm)			
	1st day	7th day	14th day	21st day
Itraconazole 100 µg/mL	< 10 mm	< 10 mm	< 10 mm	< 10 mm
O. vulgare EO 0.2 %	(-)	(-)	(-)	(-)
O. vulgare EO 0.4 %	(-)	(-)	(-)	(-)
O. vulgare EO 0.6 %	(-)	(-)	(-)	(-)
O. vulgare EO 0.8 %	(-)	(-)	(-)	(-)
O. vulgare EO 1.0 %	(-)	(-)	(-)	(-)
R. officinalis EO 0.2 %	(+)	(+)	(+)	(+)
R. officinalis EO 0.4 %	(+)	(+)	(+)	(+)
R. officinalis EO 0.6 %	(+)	(+)	(+)	(+)
R. officinalis EO 0.8 %	(+)	(+)	(+)	(+)
R. officinalis EO 1.0 %	(+)	(+)	(+)	(+)

O. vulgare OE: *Origanum vulgare* essential oil; *R. officinalis* EO: *Rosmarinus officinalis* essential oil; (-): absence of fungal growth; (+): absence of inhibition halo; < 10 mm: without inhibitory activity.

▶ **Table 4** Results of the disk-diffusion test, according to the diameter of the inhibition halos of *Origanum vulgare* L. subsp. *hirtum* and *Rosmarinus officinalis* L. essential oils in combination and reference drug on *Aspergillus fumigatus*, for 21 days.

Antifungal	Colony development assessment in days (diameter in mm)			
	1st day	7th day	14th day	21st day
Itraconazole 100 µg/mL	< 10 mm	< 10 mm	< 10 mm	< 10 mm
Association at 0.2 %	(-)	(-)	(-)	(-)
Association at 0.4 %	(-)	(-)	(-)	(-)
Association at 0.6 %	(-)	(-)	(-)	(-)
Association at 0.8 %	(-)	(-)	(-)	(-)
Association at 1.0 %	(-)	(-)	(-)	(-)

(-): absence of fungal growth; (+): absence of inhibition halo; < 10 mm: without inhibitory activity.

McFarland scale and hemocytometer count

Standardization of the fungal inoculum is essential for susceptibility testing to ensure precision and reproducibility, with the suspension adjusted to contain 1×10^5 to 2.5×10^5 CFU/mL [19, 48, 49]. To obtain the final fungal inoculum and adjust the concentration, McFarland scale techniques and Hemocytometer counting (Neubauer chamber) were used [50].

To induce the formation of young colonies and obtain the filamentous phase of the fungus (2–5 days), isolates of *A. fumigatus* were subcultured on Sabouraud dextrose agar with chloramphenicol at 28 ± 2 °C [49, 51]. Subsequently, to perform the standardization technique using the McFarland scale (DensiCHEK plus), the colonies were scraped with a sterile swab to obtain the fungal suspension, and the suspension was transferred to the sterile acrylic test tube with 5 mL of saline, checking for turbidity, to contain the correct concentration for quantification of conidia in a Neubauer chamber [50].

Disc-Diffusion Susceptibility Test

The disk-diffusion method was described by Bauer et al. in 1966 [52] and consists of defining the *in vitro* susceptibility profile of antimicrobial agents. The inoculum is streaked, and then disks soaked with antimicrobials are placed on the agar surface, checking the inhibition zone [45, 53].

In a laminar flow hood (Veco), the sterile paper discs of 6 mm diameter were prepared and organized in petri dishes according to the defined concentration [54]. For each concentration of the EOs of *O. vulgare* and *R. officinalis*, isolated and in combination (*O. vulgare* + *R. officinalis*), the EOs were solubilized in sterile distilled water and polysorbate 80 (Tween 80) at 0.2 % to facilitate absorption of EOs [55]. The itraconazole powder was weighed at a concentration of 100 µg/mL on an analytical balance (Gehaka AG 200), and the solution was solubilized in dimethylsulfoxide (DMSO) [45, 48, 49]. All solutions were vortexed (Quimis) until completely diluted, adding 25 µL to filter paper discs. These materials were dried at room temperature for 24 hours [31]. During execution of the method, Sabouraud dextrose agar medium with chloramphenicol was prepared according to the manufacturer's instructions (Kasvi), and autoclaved (Stermax) at 118 °C for 15 minutes [51]. After the standardization of the fungal inoculum and the solubilization of the medium, seeding was performed with a sterile swab inoculating the fungus, using the streak depletion technique on the plate surface. Then, using sterile forceps, 5 discs containing the isolated EOs, their combination (*O. vulgare* + *R. officinalis*) and the positive control in their respective concentrations, were deposited on the medium. The plates were kept at room temperature for 5 minutes to fix the discs in the culture medium and were subsequently taken to a glass-house (Quimis) at a temperature of 28 ± 2 °C [51, 54].

After 36 hours, with the help of a millimeter ruler, daily monitoring of petri dishes was carried out for 21 days, analyzing the growth or inhibition of the fungus and measuring the diameter of the halos observed around the discs, every 7 days. The EOs and the positive control that was considered to have inhibitory activity had an inhibition diameter ≥ 10 mm; and those without inhibitory activity showed an inhibition diameter < 10 mm [32, 56].

Susceptibility Test by Agar Dilution

In the agar dilution method, serial concentrations of the antifungal agent were distributed in several petri dishes containing the cul-

▶ **Table 5** Inhibitory activity of the essential oils of *Origanum vulgare* L. subsp. *hirtum* and *Rosmarinus officinalis* L. on the mycelial development of the fungus *Aspergillus fumigatus* and the kinetics of the mycelial growth index (MGI) of *A. fumigatus* submitted to different concentrations of these EOs, by dilution in agar for 21 days.

Antifungal	Colony development assessment in days (diameter in mm)				MGI (mm)
	1st day	7th day	14th day	21st day	
Itraconazole 100 µg/mL	(-)	(-)	(-)	(-)	0.00
O. vulgare EO 0.2 %	(-)	(-)	10 (7.75; 24)	35 (34.75; 36.5)	2.38
O. vulgare EO 0.4 %	(-)	(-)	(-)	29 (26; 33.5)	1.38
O. vulgare EO 0.6 %	(-)	(-)	(-)	(-)	0.00
O. vulgare EO 0.8 %	(-)	(-)	(-)	(-)	0.00
O. vulgare EO 1.0 %	(-)	(-)	(-)	(-)	0.00
R. officinalis EO 0.2 %	5 (5; 6)	35 (34.75; 36)	37.5 (37; 39)	37.5 (37; 39)	14.46
R. officinalis EO 0.4 %	3 (2; 4.5)	35 (34; 36)	38.5 (37.5; 39.25)	38.5 (37.5; 39.25)	12.58
R. officinalis EO 0.6 %	6.5 (3; 8)	37 (34; 39)	38.5 (36; 40)	38.5 (36; 40)	16.37
R. officinalis EO 0.8 %	5 (4; 6.75)	35 (34.75; 37.25)	38 (38; 39.25)	38 (38; 39.25)	14.52
R. officinalis EO 1.0 %	(-)	(-)	(-)	(-)	0.00

*Values are expressed as median (1º quartile; 3º quartile). *O. vulgare* OE: *Origanum vulgare* essential oil; *R. officinalis* EO: *Rosmarinus officinalis* essential oil; (-): absence of fungal growth. Significant differences ($p < 0.05$) detected by the pairwise comparisons (Durbin-Conover) are described in the text.

▶ **Table 6** Inhibitory activity of the association of the essential oils of *Origanum vulgare* L. subsp. *hirtum* and *Rosmarinus officinalis* L. on the mycelial development of the fungus *Aspergillus fumigatus* and the kinetics of the mycelial growth index (MGI) of *A. fumigatus* submitted to different concentrations of these EOs' associations, by dilution in agar for 21 days.

Antifungal	Colony development assessment in days (diameter in mm)				MGI (mm)
	1st day	7th day	14th day	21st day	
Itraconazole 100 µg/mL	(-)	(-)	(-)	(-)	0.00
Association at 0.2 %	(-)	(-)	19 (16; 24.25)	35 (33; 35.25)	3.02
Association at 0.4 %	(-)	(-)	(-)	22 (16.5; 33.5)	1.05
Association at 0.6 %	(-)	(-)	(-)	(-)	0.00
Association at 0.8 %	(-)	(-)	(-)	(-)	0.00

*Values are expressed as median (1º quartile; 3º quartile). *O. vulgare* OE: *Origanum vulgare* essential oil; *R. officinalis* EO: *Rosmarinus officinalis* essential oil; (-): absence of fungal growth. Significant differences ($p < 0.05$) detected by the pairwise comparisons (Durbin-Conover) are described in the text.

ture medium. Subsequently, the fungal samples were inoculated on the agar surface and the MIC was determined as the lowest concentration that prevents the visible growth of the fungus [57].

For this test, the Sabouraud dextrose agar medium with chloramphenicol was prepared according to the methods described above. Itraconazole powder was weighed at a concentration of 100 µg/mL on an analytical balance (Gehaka AG 200) and the solution was solubilized in dimethylsulfoxide (DMSO) [57].

In a laminar flow hood (Veco), solutions were prepared at the defined concentrations of the EOs. Fifteen mL of the culture medium was prepared for each concentration in triplicate, in which the EOs solubilized in sterile distilled water and 0.2 % Tween 80 were added, after being vortexed (Quimis) [55]. The solutions were poured into the petri dishes until complete solidification [47].

After standardization of the inoculum, mycelium discs were prepared soaked in the solution containing the fungus, and subsequently inoculated in the petri dishes. All plates were kept in a glasshouse (Quimis) at a temperature of 28 ± 2 ºC, and after 36 hours, daily monitoring was carried out, checking the development or inhibition of the fungus, using positive and negative control as the growth pattern [7, 47]. The evaluation of the antifungal activity of the EOs and the positive control was verified through the development of the colonies (diameter in millimeters), with the measurement of mycelium with the help of a millimeter ruler every 7 days, for 21 days, calculating the average of the diameter of the colonies to obtain the kinetics of the mycelial growth index (MGI) [47, 56]. The MGI calculation was performed according to the formula presented below [56]:

$$MGI = \frac{C1}{D1} + \frac{C2}{D2} + \ldots \frac{Cn}{Dn}$$

Where:

MGI = mycelial growth index
C = colony size
D = days, when measurements were taken

Statistical analysis

Statistical analysis was carried out using R software, version 3.6. The continuous variables were tested for normal distribution with Shapiro-Wilk. Differences among the analyzed concentrations and those over time were investigated through the non-parametric Repeated Measures ANOVA (Friedman Test; data not normally distrib-

uted), followed by the Pairwise Comparisons (Durbin-Conover). Values of $p < 0.05$ were considered statistically significant.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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