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THE POTENTIAL OF *ORIGANUM VULGARE* L. (LAMIACEAE) ESSENTIAL OIL IN INHIBITING THE GROWTH OF SOME FOOD-RELATED *ASPERGILLUS* SPECIES

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ABSTRACT

Origanum vulgare L. (Lamiaceae) has been currently known for their interesting antimicrobial activity being regarded as alternative antimicrobial for use is food conservation systems. This study aimed to evaluate the effectiveness of *O. vulgare* essential oil in inhibiting the growth of some food-related *Aspergillus* species (*A. flavus, A. parasiticus, A. terreus, A. ochraceus, A. fumigatus* and *A. niger*). The essential oil revealed a strong anti-*Aspergillus* property providing an inhibition of all assayed mould strains. MIC values were between 80 and 20 µL/mL being found a MIC_{50} of 40 µL/mL. The essential oil at concentration of 80 and 40 µL/mL provided a fungicidal effect on *A. flavus*, *A. fumigatus* and *A. niger* noted by a total inhibition of the radial mycelial growth along 14 days of interaction. In addition, the essential oil was able to inhibit the mould spores germination when assayed at concentrations of 80 and 40 µL/mL. Our results showed the interesting anti-*Aspergillus* activity of *O. vulgare* essential oil supporting their possible use as anti-mould compound in food conservation.

Key-words: *O. vulgare*, essential oil, *Aspergillus*

INTRODUCTION

Moulds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes, carried and disseminated by wind and air currents, as well as can be spread by insects, rodents, and other animals. The metabolic activities accompanying the growth and mould development decomposes organic substrate ensuring the recycling of elements that comprise organic matters. Food products, being organic substances and containing essential nutrients, are very suitable substrates for the mould growth (13,15,28). Because their powerful arsenal of hydrolytic enzymes, moulds can cause a high degree of deterioration when present in/on foods and can be responsible for considerable economic losses. However, some people see actually to prefer, on the basis of flavor, foods with a touch of mouldiness, while mould cultures have been frequently used in the preparation of various traditional fermented foods (14,21).

Besides the possible food decaying caused by moulds and ultimate changes in it nutritional and organoleptical characters, the moldiness in foodstuffs is toxicologically significant since the mould species growing on such products is known as potentially mycotoxicogenic (4,15). Mycotoxins are toxic metabolites produced by filamentous fungi that have been detected in several food commodities. Levels of mycotoxins and mycotoxicogenic moulds, which can cause risk to population, are refused by consumers and many countries have set regulations in various agricultural foods. The consumption of mouldy products can cause human or animal mycotoxicoses, and more important some mycotoxins are potent carcinogens (3,14,23,36).

Aspergillus genus, which presents species inserted in the group of infesting living plants/field fungi (e.g. *A. flavus*) and

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infesting stored food products/storage fungi (e.g. *A. parasiticus, A. ochraceus, A. fumigatus, A. chevalieri* and *A. clavatus*), is responsible for many causes of food contaminations all over the world (1, 36). Aflatoxins-B_1, B_2, G_1, G_2 (produced by *A. flavus* and *A. parasiticus*), aspergillic acid (produced by *A. flavus*), hidroxiaspergillic acid (produced by *A. flavus*), ascladiol (produced by *A. clavatus*), gliotoxin (produced by *A. fumigatus*, *A. chavalieri* and *A. terreus*), austamide (produced by *A. usutus*), ochratoxins (produced by *A. ochraceus*), oxalic acid (produced by *A. flavus* and *A. glaucus*), terreic acid (produced by *A. terreus*) are some mycotoxins produced by *Aspergillus* species in foods when exposed to suitable conditions (13,15,17).

Chemicals are used to inhibit the fungal growth in/on foods, however the negative consumer perception of chemical preservatives drives attention toward natural alternatives, so that many researches worldwide have been carried out in order to evaluate the effectiveness of many plant products in inhibiting the growth of spoiling and/or pathogen food-related microorganisms. *Origanum vulgare* L., Lamiaceae family, is widely known as a very versatile plant with many therapeutic properties (diaphoretic, carminative, antispasmodic, antiseptic, tonic) being applied in traditional medicine systems in many countries (26,27). It has been widely used in agricultural, pharmaceutical and cosmetic industries as culinary herb, flavoring substances in food products, alcoholic beverages and perfumery for its spicy fragrance (2,12,20). Although, some researchers have found antimicrobial activity in *Origanum vulgare* L. (6,9,19,30), there is a lack of information about their effect on the kinetic of the mycelial growth and mould spores germination of *Aspergillus* species.

The aim of this study was to evaluate the effect of *Origanum vulgare* L. essential oil on some aspects related to the growth of some *Aspergillus* species recognized as potential food-contaminating.

MATERIAL AND METHODS

Essential oil

Origanum vulgare L. essential oil was supplied by Ferquima Ind. Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil) and its quality parameters: appearance, color, purity, odor, density - 20ºC, refraction index - 20ºC were described in an accompanying technical report. This provider produces and commercializes essential oils on an industrial scale. The essential oil was assayed at concentrations of 320, 160, 80, 40, 20, 10 and 5 µL/mL and the solutions were prepared according to Souza *et al.*, (36).

Moulds strains

Aspergillus fumigatus (ATCC-16913 and ATCC-40640), *A. niger* (P-03 and LM-257), *A. flavus* (ATCC-16013 and LM-247), *A. parasiticus* (ATCC-15517 and NRRL-2999), *A. terreus* (UP-03 and ATCC-7860) and *A. ochraceus* (ATCC-7860 and LM-06) strains were used as test microorganisms. These strains were taken from the Microorganisms Collection, Laboratory of Mycology, Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. Stock cultures were maintained on Sabouraud agar (SA) slants and stored in a refrigerator.

Minimum Inhibitory Concentration - MIC

The essential oil MIC was found by a qualitative method using the solid medium diffusion procedure (31,32). For this, 1 mL of mould homogenous suspension (approximatelly 10^6 spores/mL) prepared according to Rana *et al.* (22) was uniformly spread on sterile SA Petri dishes. After inoculum absorption by SA, wells were made using sterile glass stems (diameter 6 mm) which were filled with 50 µL of the essential oil solution at different concentrations. The incubation period was 7-10 days at 25ºC. At the end of the incubation period, the MIC was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm.

Control was carried out with amphotericin B (100 µg/mL) and ketoconazole (50 µg/mL) by solid medium diffusion procedure using filter paper discs (Cecon, diameter 6 mm) (5). Also, it was carried out the control of viability of the assayed mould strains by observing their capability of growing on SA without adding essential oil or standard antifungical. Each assay was performed twice and the results were expressed as the average of the two repetitions.

Mycelial growth inhibition

Inhibition of the dematiaceous mould mycelial growth was determined using the poisoned substrate technique by the daily measure of the radial mycelial growth on SA added of the essential oil in an amount adjusted to provide a final concentration similar to the MIC previously found (1,10). For this, a 2 mm plug taken from a 10-days-old mould culture cultivated on SA slants were placed on the center of the sterile SA Petri dishes added of the essential oil prior to the assay and incubated at 25-27ºC for 10 days. At different intervals (0, 2, 4, 6, 8, 10, 12 and 14 days) after incubation, the mould radial mycelial growth was measured (mm) using calipers. Control included in this assay was the observation of the mould radial growth on SA without adding essential oil, and added of ketoconazole (50 µg/mL). Each assay was performed twice and the results were expressed as the average of the two repetitions.

Spore germination assay

Different concentrations of the essential oil were tested for spore germination of the assayed mould strains. Aliquots of 0.2 mL of the essential oil solutions at different concentration were mixed with 0.2 mL of the mould spores suspension (approximatelly 10^5 spores/mL) followed by shaking using Vortex for 30 seconds. 0.1 mL of the mixture was placed on separated glass slides which

were incubated in a moist chamber at 25 ±2ºC for 24 hours. At the end of the incubation period, each slide was fixed with lacto-phenol-cotton blue stain and observed under the microscope for spore germination. About 200 spores were counted and the per cent spore germination was calculated in comparison with the control assay where the essential oil was replaced for sterile distilled water (22,29). Each assay was performed twice and the results were expressed as the average of the two repetitions.

Statistical analysis

Statistical analysis was performed to determine significant differences ($P<0.05$) by Tukey test in the mould mycelial radial growth assays. For this was used Sigma stat 2.03 computer program.

RESULTS AND DISCUSSION

In the last years the scientific literature in food science and technology has emphasized the antimicrobial activity of essential oils regarding their use in the named food bioconservation systems. Food bioconservation is a widely accepted system being referred as a natural procedure able to provide the extension of the shelf-life and food microbial safety (25,24,34). In this renewed interest, *O. vulgare* essential oil has been known as interesting source of antimicrobial compounds to use in food conservation (10,20,32,33).

Results of the inhibitory activity of *O. vulgare* essential oil on the growth of 12 *Aspergillus* species strains in solid medium are shown in Table 1. Fungal growth inhibition was noted in all assayed *Aspergillus* species being found a dose dependent anti-mould activity. On the basis of mould growth inhibition zones, various concentrations (320 – 40 µL/mL) of *O. vulgare* essential oil showed a very strong anti-mould activity. At concentrations from 320 to 80 µL/mL the essential oil inhibited all assayed strains providing the development of wide growth inhibition zones (10 – 25 mm). On the other hand, all tested moulds strains were resistant to concentration of 10 and 5 µL/mL. 40 µL/mL was the MIC_{50} (concentration of the essential oil causing a growth inhibition of 50% or more of the assayed strains) found to *O. vulgare* essential oil. The lowest MIC values (10 µL/mL) were found at interactions with *A. niger* P-03 and *A. flavus* LM-247.

In some assayed concentrations (320 to 40 µL/mL) the essential oil provided the development of fungal growth inhibition zones with diameter equal to or higher than the ones found to the tested standard antifungicals (amphotericin B and ketoconazole). Amphotericin B showed a weak capacity in inhibiting the mould growth with inhibition zones showing diameters between 0 and 12 mm.

Study of kinetic of mould growth (Fig. 1, 2 and 3) assessed by radial mycelial growth (mm) measure along different time intervals revealed a fungicidal effect of *O. vulgare* essential oil when assayed in the 80 (MIC_{50} value) and 40 µl/mL on *A. flavus*, *A. fumigatus* and *A. parasiticus*. The essential oil provided significant ($P<0.05$) reduction in the mycelial mould growth when compared with the control assay and ketoconazole. *O. vulgare* provided 100% of lethal effect already after 2 days of interactions and no mycelial growth occurred in the later times. These

Table 1. Inhibitory effect of *O. vulgare* essential oil on the growth of some *Aspergillus* species (results expressed in millimeters of mould growth inhibition zones).

Mould strains	O. vulgare essential oil (µL/mL)							Control		
	320	160	80	40	20	10	5	Amph B [a] (100 µg/mL)	Ketoc [b] (50 µg/mL)	Viab [c]
A. fumigatus ATCC-16913	25	20	20	8	0	0	0	8	18	+
A. fumigatus ATCC-40640	18	15	12	7	0	0	0	0	10	+
A. niger P-03	24	17	15	12	10	0	0	12	10	+
A. niger LM-257	27	25	21	13	0	0	0	8	10	+
A. flavus ATCC-16013	27	24	20	10	0	0	0	7	22	+
A. flavus LM-247	30	22	20	12	10	0	0	7	15	+
A. parasiticus ATCC-15517	16	15	14	12	0	0	0	8	20	+
A. parasiticus NRRL-2999	18	14	12	8	0	0	0	7	20	+
A. terreus UP-03	23	20	17	15	8	0	0	0	15	+
A. terreus ATCC-7860	24	20	17	10	7	0	0	0	20	+
A. ochraceus ATCC-7860	18	14	10	8	0	0	0	7	12	+
A. ochraceus LM-06	24	18	14	10	8	0	0	0	12	+

[a] amphotericin B; [b] ketoconazole; [c] strain viability: ability of the strain to grow in Sabouraud agar without adding essential oil or synthetic antibiotic.

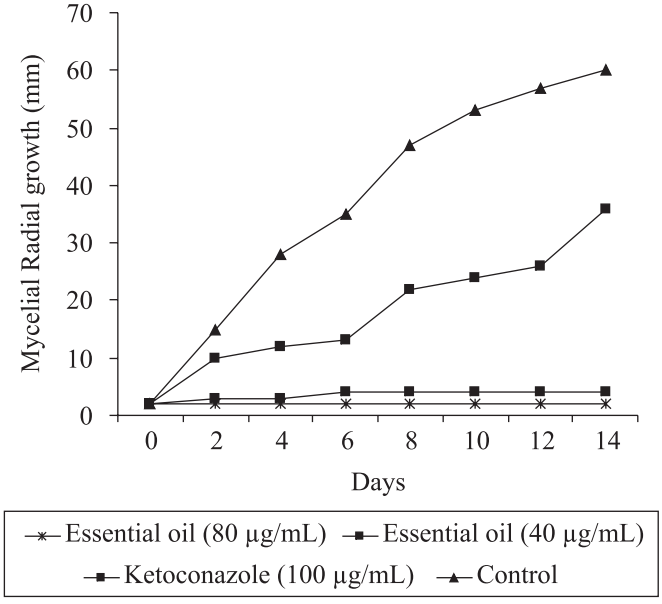

Figure 1. Effect of *O. vulgare* essential oil and ketoconazole on the radial mycelial growth kinetic of *A. flavus* LM-247.

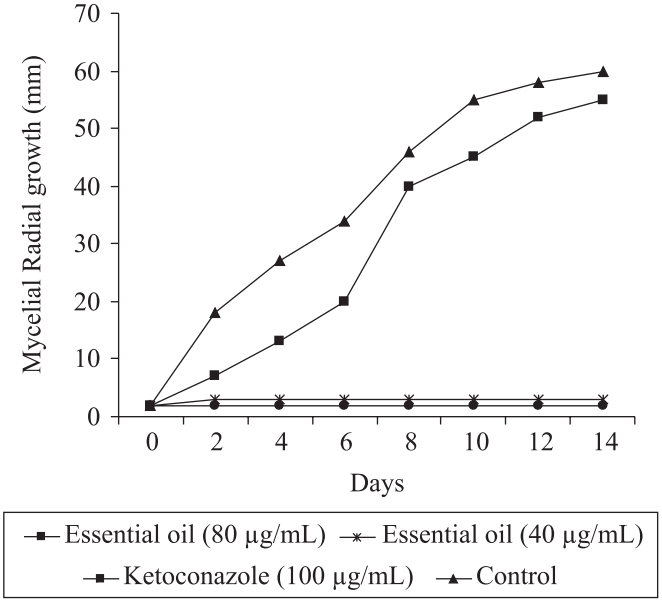

Figure 2. Effect of *O. vulgare* essential oil and ketoconazole on the radial mycelial growth kinetic of *A. fumigatus* ATCC-40640.

findings show an intense fumigant property of *O. vulgare* essential oil being a fast and steady rate of mycelial growth inhibition a characteristic of the essential oil. Only in the assay with *A. flavus* LM-247 was noted a slow and small increase (2 to

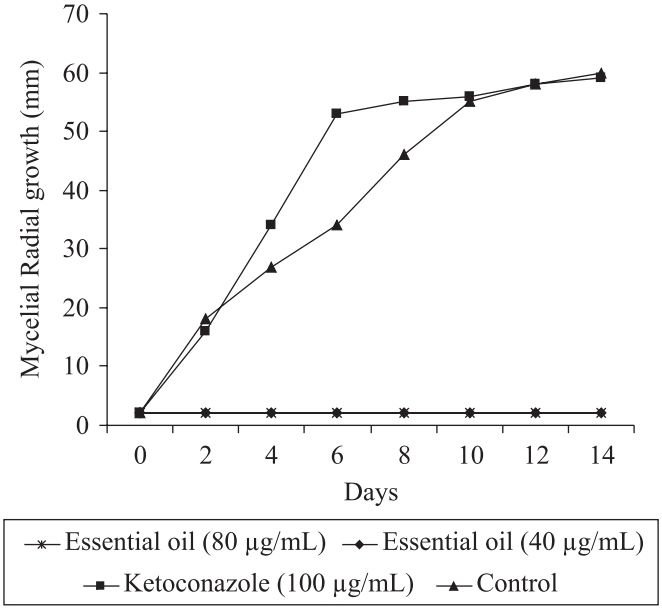

Figure 3. Effect of *O. vulgare* essential oil and ketoconazole on the radial mycelial growth kinetic of *A. niger* P-03.

4 mm) in the mycelial growth up to 6 days of exposure when the essential oil was assayed a t concentration of 40 µL/mL.

Ketoconazole was included in the mould growth kinetic assay because no assayed mould presented resistance to it in the MIC assay. Ketoconazole showed no significant ($P<0.05$) inhibition of the radial mycelium growth. All tested mould presented a progressive mycelial growth when in exposure to ketoconazole along the evaluated time intervals.

The results of the effect of *O. vulgare* essential oil at concentrations of 40 and 80 µL/mL on the spore germination of *A. parasiticus*. As can be noted the essential oil exhibited a strong inhibition of spore germination of all tested fungi by both assayed concentrations. A 100% inhibition was found at 80 µL/mL on *A. niger*, while at 40 µL/mL concentration the inhibition was always over 90%. In addition, it was noted that those spores which germinated in presence of the essential oil produced smaller germ tubes (early growing hyphae) as compared with the control assay (data not shown). In agreement with earlier researches (22,29) the inhibition of spore germination caused by *O. vulgare* essential oil was in a dosage response manner.

Earlier studies have noted the effectiveness of some essential oils in inhibiting the growth and mycotoxins synthesis of some food-related moulds in laboratorial media and foodstuffs. Basílico and Basílico (3) and Velluti *et al.* (35) found significant inhibitory effect of *O. vulgare* essential oil on the growth of *Fusarium proliferatum* and *Aspergillus ochraceus* and ochratoxin A and fumonisin B_1, respectively. On the other hand,

Table 2. Inhibition of *O. vulgare* essential oil on spore germination of *Aspergillus* species (results expressed in percent of spore germination inhibition in comparison with the control assay).

Moulds	*O. vulgare* essential oil (concentrations)	
	40 µL/mL	80 µL/mL
A. flavus LM-247	91 %	100 %
A. fumigatus ATCC-40640	93 %	100 %
A. niger P-03	100 %	100 %

Marín *et al.* (17) noted a poor efficacy of *O. vulgare* essential oil to inhibit the growth of *Fusarium graminearum* and production of zearalenone and deoxynivalenol.

Essential oils presenting high amount of phenolic compounds have showed the greatest anti-mould activities. Some researchers have reported high content of phenolic compounds in *O. vulgare* essential oil, mostly thymol and carvacrol, which are probably mainly responsible for its prominent antimicrobial effect. *o*-cymene, *p*-cymene, á-pinene, myrcene, ã-terpinene, canfene, limonene, trans-caryophyllene, borneol, linalool and cineole are some minority compounds found in *O. vulgare* essential oil (19,32).

Phenolic compounds, in appropriate concentration and prolonged application, are effective against several microorganisms (8). Their mechanism of antimicrobial activity is related to protoplasmic poisoning, disruption of microbial cell wall, and precipitation of cell protein (7, 8). In addition, the antimicrobial activity of these compounds has been attributed to the presence of an aromatic nucleus and an OH group known to be reactive and to form hydrogen bonds with active sites of target enzymes (35).

It has been found that essential oils with anti-mould effectiveness is able to consistently cause morphological changes in *Aspergillus* species including lack of sporulation, loss of pigmentation, aberrant development of conidiophores (flattened and squashed) and distortion of hyphaes (budding, lack of cytoplasm, swelling anomalous apex bifurcation) (11,16,23,29). These findings suggested that the mode of antifungal activity of essential oils could include an attack on the cell wall and retraction of the cytoplasm in the hyphae ultimately resulting in death of the mycelium. In addition, it was also related to the interference of the essential oil components in enzymatic reactions of wall cell synthesis, which affects the fungal growth and morphogenesis.

The results found in this study showed a fungicidal activity of *O. vulgare* essential oil providing interesting information about rapidity and stability of its anti-*Aspergillus* property. It also showed that the growth and survival of spoiling and food-related *Aspergillus* species in foods could be controlled by the use of essential oils, particularly, *O. vulgare* essential oil. Regarding our results, further researches are being designed to assess the interference of *O. vulgare* essential oil in the mycotoxin synthesis of some toxicogenic *Aspergillus* species.

RESUMO

Potencial do óleo essencial de *Origanum vulgare* L. (Lamiaceae) em inibir o crescimento de algumas cepas de Aspergillus de interesse em alimentos

Origanum vulgare L. (Lamiaceae) tem sido atualmente reconhecido por sua intensa atividade antimicrobiana sendo considerado como fonte de compostos antimicrobianos alternativos para uso em sistemas de conservação de alimentos. Este estudo objetivou avaliar a efetividade do óleo essencial de *O. vulgare* em inibir o crescimento de algumas espécies de *Aspergillus* (*A. flavus, A. parasiticus, A. terreus* and *A. fumigatus*) de interesse em alimentos. O óleo essencial revelou uma forte atividade atni-*Aspergillus* provocando a inibição de todas as cepas fúngicas ensaiadas. Os valores de MIC estiveram entre 80 e 20 µL/mL sendo encontrado uma MIC_{50} de 40 µL/mL. O óleo essencial nas concentrações de 80 e 40 µL/mL causou um efeito fungicida sobre *A. flavus*, *A. fumigatus* e *A. niger* notado por uma total inibição do crescimento micelial radial ao longo de 14 dias de interação, bem como foi capaz de inibir a germinação de esporos destas cepas fúngicas. Nossos resultados mostram a intensa atividade anti-*Aspergillus* do óleo essencial de *O. vulgare* suportando o seu possível uso como antifúngico em sistemas de conservação de alimentos.

Palavras-chave: *O. vulgare*, essential oil, *Aspergillus*

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