ANTIFUNGAL ACTIVITIES OF THYME, CLOVE AND OREGANO ESSENTIAL OILS

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ABSTRACT

*The antifungal potential of essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) was determined. To establish this antifungal potential, two molds related to food spoilage,* Aspergillus niger *and* Aspergillus flavus, *were selected. The agar dilution method was employed for the determination of antifungal activities. The three essential oils analyzed presented inhibitory effects on both molds tested. Oregano essential oil showed the highest inhibition of mold growth, followed by clove and thyme.* Aspergillus flavus *was more sensitive to thyme essential oil than* A. niger. *Clove essential oil was a stronger inhibitor against* A. niger *than against* A. flavus.

PRACTICAL APPLICATIONS

The use of essential oils from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) as antifungal agents will be suitable for applications on the food industry. They can be used as growth inhibitors of *Aspergillus niger* and *Aspergillus flavus*, two of the more important molds of foodborne diseases and/or food spoilage. The main reason for their suitability is their natural origin, which consumers find comforting and which is beneficial for the environment, and the very low risk that pathogens will develop resistance to the mixture of components that make up the oils with

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their apparent diversity of antifungal mechanisms. These beneficial characteristics could increase food safety and shelf life.

INTRODUCTION

In many traditional products (meats, fish and dairy products) from the Mediterranean basin, the use of spices is common, whether in the elaboration of food or in its preservation. Products such as pepper sausage, where pepper may be incorporated in the meat or added to the surface, and cheese which includes a variety of spices, are widely consumed. In both cases, sensorial factors (taste and smell) are of greater concern than other factors (technological, shelf life, etc.).

Furthermore, in this type of "homemade" or traditional product, the use of chemical agents is frowned upon by consumers. However, as any other type of food, these traditional products are exposed to chemical and/or microbiological alteration and subsequent adverse economic consequences for the manufacturer.

Another serious problem about the use of certain chemical agents used in the protection against such alterations is the development of resistance to them by the fungi concerned. Furthermore, the application of higher concentrations of chemicals in an attempt to overcome this problem increases the risk of high-level toxic residues in the products (Daferera *et al*. 2003).

A variety of microorganisms can lead to food spoilage in the food industry. So far, many pathogenic molds, such as *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp., have been reported as the causal agents of foodborne diseases and/or food spoilage (Betts *et al.* 1999). Recently, there has been considerable interest expressed in extracts and essential oils from aromatic plants with antimicrobial activities for controlling pathogens and toxin-producing microorganisms in foods (Soliman and Badeea 2002; Tepe *et al.* 2005).

Numerous studies have documented the antifungal properties of plant essential oils (Bouchra *et al*. 2003; Daferera *et al*. 2003; Sokmen *et al*. 2004). These properties are caused by many active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarins and curcumines (Tepe *et al.* 2005). Because of health and economic considerations, the search for antifungal agents is extensive (Paster *et al*. 1995). Natural plant extracts may provide an alternative way to protect foods or feeds from fungal contamination.

The specific objective of this work was to determine the effectiveness of using the essentials oils from oregano (*O. vulgare* L.), thyme (*T. vulgaris* L.) and clove (*S. aromaticum* L.) in combating the growth of some molds related to food spoilage, namely *A. niger* and *A. flavus*.

MATERIALS AND METHODS

Essential Oils

The essential oil of thyme (*T. vulgaris*), ref. F71180L, was obtained by steam distillation from leaves, stem and flowers; its density at 20C was 0.944 g/mL, the refraction index at 20C was 1.507, while the boiling point was higher than 100C. Clove (*S. aromaticum*), essential oil ref. F08568L, was obtained by steam extraction from the fruit; its density at 20C was 1.093 g/mL, and the refraction index at 20C was 1.478, while the boiling point was higher than 70C. Oregano (*O. vulgare*), essential oil ref. F70900L, was obtained by steam extraction from flowers; its density at 20C was 0.938 g/mL, and the refraction index at 20C was 1.509 while its boiling point was higher than 100C. All essential oils were purchased from Ravetllat Aromatics (Barcelona, Spain).

Antifungal Activity

Microbial Strains. The essentials oils were individually tested against a panel of molds, consisting of *A. niger* CECT 2091 and *A. flavus* CECT 2685. Both species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia.

Agar Dilution Method. The food pathogenic fungi were tested by the agar dilution method (Fraternale *et al.* 2003), with some modifications, in the appropriate culture medium (potato dextrose agar; Oxoid, Basingstoke, Hampshire, England). The oils tested were added to the culture medium at a temperature of 40–45C, and then poured into petri dishes (10 cm in diameter). Concentrations of 2, 4, 6 and 8 µL essential oils/18 mL culture medium (0.11, 0.22, 0.33 and 0.44 µL/mL culture medium, respectively) were tested for thyme, clove and oregano. The molds were inoculated as soon as the medium had solidified. A disk (9 mm in diameter; Schlinder & Schuell, Dassel, Germany) of mycelial material, taken from the edge of 5-day-old fungi cultures, was placed at the center of each petri dish. The petri dish with the inoculum was then placed to incubate at 25C. The efficacy of treatment was evaluated each day during 8 days by measuring the diameter of the fungus colonized. The values were expressed in millimeters diameter per day. All tests were performed in triplicate.

Statistical Analysis

Each parameter was tested in triplicate. Conventional statistical methods were used to calculated means and standard deviations. Statistical analysis

one-way (analysis of variance [ANOVA]) was applied to the data to determine differences ($P < 0.05$). To ascertain significant differences between the levels of the main factor, Tukey's test was applied between means (Afifi and Azen 1979). ANOVAs were made with the following factor: time (9 levels; 0, 1, 2, 3, 4, 5, 6, 7 and 8 days) for each concentration and essential oil. Another statistical ANOVA (one-way ANOVA) was applied using the following factor: essential oils: (3 levels; thyme, clove and oregano). Statistical data analysis was undertaken using the statistical package Statgraphics plus 2.0.

RESULTS AND DISCUSSION

The antifungal activities of the essential oils of thyme, clove and oregano on the molds *A. niger* and *A. flavus* are shown in Tables 1 and 2, respectively. Table 3 shows the values of percent growth reductions of the same essentials oils at day 8.

In the case of *A. niger*, it can be seen that thyme essential oil reduced fungal growth when used at 2 µL/18 mL culture medium, although the total inhibition was only attached when 8 µL was used.

The total inhibition was also obtained with the 8 and 6 µL/18 mL culture medium concentrations of clove essential oil, while the 4 and 2 µL concentrations achieved lower reductions, although both reduction values were higher than those achieved with thyme essential oil.

When oregano essential oil was used, the 2 µL concentration reduced mycelium growth, total inhibition being achieved in this case with the 4, 6 and 8 µL/18 mL culture medium concentrations.

Oregano essential oil, then, provided the highest degree of inhibition of *A. niger* growth because at the lowest concentration, it was a more potent inhibitor of growth than the other essential oils studied. Only clove at the 4 µL concentration showed a slightly higher degree than oregano at 2 µL. The next essential oil in order of its power to inhibit *A. niger* growth was clove, thyme being the poorest inhibitor.

In the case of *A. flavus*, thyme essential oil reduced mycelial growth at 2, 4 and 6 µL. Inhibition was total at 8 µL. These percent reduction values are higher than those obtained with *A. niger*, meaning that *A. flavus* was more sensitive to thyme essential oil than *A. niger.*

With clove, mycelial growth was completely inhibited at 6 and 8 µL, as it was in the case of *A. niger*. Clove essential oil was a stronger inhibitor of *A. niger* than of *A. flavus.*

In the case of the essential oil of oregano, growth was reduced at 2 µL (by 57%) and totally so by all the higher concentrations. Such growth reduction values showed similar values to those obtained with *A. niger*.

TABLE 1.
ANTIFUNGAL ACTIVITY OF THYME, CLOVE AND OREGANO ESSENTIAL OILS USING AGAR DILUTION METHOD UPON *ASPERGILLUS NIGER*

	Diameter (mean and SD $n = 3$) of mycelial growth (mm) including disk diameter of 9 mm								
	0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
Control	9.0 ± 0.0	13.46 ± 0.23	27.11 ± 0.26	35.32 ± 0.16	42.77 ± 0.29	49.97 ± 0.22	54.67 ± 0.28	62.51 ± 0.33	68.88 ± 0.30
Thyme 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	14.15 ± 0.10^{bL}	24.05 ± 0.22^{cL}	27.85 ± 0.20^{dL}	35.32 ± 0.26^{eL}	43.25 ± 0.35^{fL}	49.61 ± 0.20^{gL}	54.31 ± 0.22^{hL}
Thyme 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	12.31 ± 0.10^{bA}	17.52 ± 0.26^{cA}	23.38 ± 0.30^{dA}	31.92 ± 0.27^{eA}	39.86 ± 0.41^{fA}	44.61 ± 0.25^{gA}	48.94 ± 0.44^{hA}
Thyme 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	11.02 ± 0.12^{bX}	14.31 ± 0.32^{cX}	18.63 ± 0.16^{dX}	24.15 ± 0.26^{eX}	29.94 ± 0.39^{fX}	36.72 ± 0.17^{gX}	41.53 ± 0.24^{hX}
Thyme 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Clove 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	10.07 ± 0.05^{bM}	13.68 ± 0.09^{cM}	16.55 ± 0.22^{dM}	21.98 ± 0.26^{eM}	27.21 ± 0.19^{fM}	31.77 ± 0.18^{gM}	35.81 ± 0.19^{hM}
Clove 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aB}	9.00 ± 0.00^{aB}	13.18 ± 0.24^{dB}	17.31 ± 0.26^{eB}	21.02 ± 0.34^{fB}	23.38 ± 0.24^{gB}	26.51 ± 0.22^{hB}
Clove 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}
Clove 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Oregano 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	10.56 ± 0.13^{bN}	11.89 ± 0.19^{cN}	14.86 ± 0.28^{dN}	18.21 ± 0.26^{eN}	21.98 ± 0.32^{fN}	25.78 ± 0.22^{gN}	29.74 ± 0.27^{hN}
Oregano 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aB}	9.00 ± 0.00^{aB}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}
Oregano 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}
Oregano 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}

Values followed by the same small letter within the same line are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (L–N) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (A–C) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (X–Z) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (R–T) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.

TABLE 2.
ANTIFUNGAL ACTIVITY OF THYME, CLOVE AND OREGANO ESSENTIAL OILS USING AGAR DILUTION METHOD UPON *ASPERGILLUS FLAVUS*

	Diameter (mean and SD $n = 3$) of mycelial growth (mm) including disk diameter of 9 mm								
	0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
Control	9.00 ± 0.00	9.67 ± 0.20	25.39 ± 0.38	40.12 ± 0.31	51.53 ± 0.34	67.93 ± 0.42	82.76 ± 0.25	88,43 ± 0.16	90.00 ± 0.00
Thyme 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	15.73 ± 0.36^{bL}	20.04 ± 0.38^{cL}	26.89 ± 0.41^{dL}	34.81 ± 0.30^{eL}	41.01 ± 0.42^{fL}	50.42 ± 0.49^{gL}	57.94 ± 0.43^{hL}
Thyme 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	13.96 ± 0.52^{bA}	17.89 ± 0.45^{cA}	23.54 ± 0.33^{dA}	30.61 ± 0.40^{eA}	36.94 ± 0.40^{fA}	44.62 ± 0.25^{gA}	51.04 ± 0.36^{hA}
Thyme 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	11.63 ± 0.11^{bX}	14.72 ± 0.28^{cX}	18.93 ± 0.45^{dX}	24.91 ± 0.21^{eX}	31.14 ± 0.30^{fX}	37.82 ± 0.31^{gX}	45.82 ± 0.25^{hX}
Thyme 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Clove 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	23.4 ± 0.41^{bM}	28.27 ± 0.36^{cM}	38.05 ± 0.29^{dM}	45.82 ± 0.27^{eM}	51.00 ± 0.36^{fM}	56.91 ± 0.41^{gM}	63.83 ± 0.28^{hM}
Clove 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	18.39 ± 0.32^{bB}	25.46 ± 0.30^{cB}	34.15 ± 0.24^{dB}	41.78 ± 0.53^{eB}	45.94 ± 0.28^{fB}	49.63 ± 0.27^{gB}	55.23 ± 0.28^{hB}
Clove 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± .00^{aY}	9.00 ± 0.00^{aY}
Clove 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}
Oregano 2 µL	9.00 ± 0.00^{aL}	9.00 ± 0.00^{aL}	11.01 ± 0.08^{bN}	15.65 ± 0.21^{cN}	19.67 ± 0.13^{dN}	24.53 ± 0.33^{eN}	29.31 ± 0.38^{fN}	33.51 ± 0.39^{gN}	38.72 ± 0.16^{hN}
Oregano 4 µL	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aA}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}	9.00 ± 0.00^{aC}
Oregano 6 µL	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aX}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	9.00 ± 0.00^{aY}	90.0 ± 0.00^{aY}	9.00 ± 0.00^{aY}
Oregano 8 µL	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}	9.00 ± 0.00^{aR}

Values followed by the same small letter within the same line are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (L–N) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (A–C) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (X–Z) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.
Values followed by the same letter (R–T) within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple-range test.

TABLE 3.
REDUCTION PERCENTAGE VALUES OF THYME, CLOVE AND OREGANO ESSENTIAL OILS UPON THE GROWTH OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS NIGER*

% Growth reduction

	Concentration (μL/18 mL)	Thyme	Clove	Oregano
A. niger	2	21.2	48.0	56.8
	4	29.0	61.5	100.0
	6	39.7	100.0	100.0
	8	100.0	100.0	100.0
A. flavus	2	35.6	29.1	57.0
	4	43.3	38.6	100.0
	6	49.1	100.0	100.0
	8	100.0	100.0	100.0

Sokmen *et al*. (2004) demonstrated the capacity of thyme essential oil at 10 μL to inhibit the growth of molds such as *Alternaria* spp., *A. flavus*, *Fusarium* spp. and *Penicillium* spp. This antifungal capacity of thyme essential oil has also been demonstrated by Montes and Carvajal (1998) and Basilico and Basilico (1999) on fungi such as *A. flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus fumigatus* and *Fusarium* spp. Other authors, too, including El-Maraghy (1995) and Inouye *et al*. (2000), confirmed these results. According to Soliman and Badeea (2002), this antifungal effect might be caused by the β-pinene content of thyme essential oil because it can reach values of 29.9–37.6%.

Paster *et al*. (1995) demonstrated the antifungal activity of oregano essential oil at concentrations of 2 and 2.5 μL/L on the mycelium and spores of *A. niger*, *A. flavus* and *A. ochraceus*, findings that agree with those of other authors, including Baratta *et al*. (1998) and Bouchra *et al*. (2003), who also showed that the antifungal activity of oregano essential oil on *A. niger* is much stronger than other essential oils such those of rosemary or sage. It is well established (Arnold *et al*. 2000; Veres *et al*. 2003) that carvacol is the major component of oregano, and many authors (Sokovic *et al*. 2002; Lopez *et al*. 2005) have attributed the antifungal properties of oregano to this compound. Its action mechanism has not been firmly established, although interaction with the cell membrane of the pathogen is thought to be likely (Veres *et al*. 2003).

The other essential oil to show a high antifungal capacity on the molds *A. niger* and *A. flavus* was clove, and other authors such as Chalfoun *et al*. (2004) and Kong *et al*. (2004) working with molds of the genera *Aspergillus* spp. and *Penicillium* spp. corroborate these findings. Some authors (Kong *et al*. 2004; Chami *et al.* 2005) have attributed this inhibitory capacity to eugenol, the major component of clove essential oil.

Possible modes of action of essential oil constituents (phenolic and terpenes) have been reported in different reviews (Davidson 2001). However, the mechanisms have not been completely elucidated. Prindle and Wright (1977) mentioned that the effect of phenolic compounds is concentration dependent. At low concentrations, phenols affect enzyme activity, especially of those enzymes associated with energy production; at greater concentrations, they cause protein denaturation. The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds to alter microbial cell permeability, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in their structure and functionality (Fung *et al.* 1977). Lis-Balchin and Deans (1997) reported that strong antimicrobial activity could be correlated with essential oils containing a high percentage of monoterpenes, eugenol, cinnamic aldehyde and thymol. Conner and Beuchat (1984) suggested that the antimicrobial activity of the essential oils of herbs and spices or their constituents such as thymol, carvacrol, eugenol, etc., could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds. Nychas (1995) indicated that phenolic compounds could denature the enzymes responsible for spore germination or interfere with the amino acids involved in germination. Once the phenolic compounds have crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity. Davidson (2001) reported that the exact cause–effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined, although it seems that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material functionality.

Several studies have attempted to determine the efficacy of extracts from selected plants as antimicrobial and antifungal agents (Lopez *et al.* 2000). Some studies have shown that specific essential oils and phenolic compounds can control the growth rate and spore germination time of spoilage fungi (Hope *et al.* 2003).

The antifungal activity of essential oils is mainly attributable to their major components although the possibility of other phenomena, such as synergy or antagonism with minor components, must also be borne in mind (Daferera *et al.* 2003).

CONCLUSIONS

From the reported results, it can be concluded that the tested essential oils exhibited, *in vitro*, broad spectrum of antifungal activity against *A. niger* and

A. flavus. So, these essential oils can be used as antifungal agents, being the main reason for their suitability, their natural origin, which consumers find comforting and which is beneficial for the environment, and the very low risk that pathogens will develop resistance to the mixture of components that make up the oils with their apparent diversity of antifungal mechanisms.

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