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Effectiveness of *Origanum vulgare* L. essential oil to inhibit the growth of food spoiling yeasts

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Abstract

Origanum vulgare L. has been known as having many therapeutic properties and its antimicrobial activity has currently received a renewed interest. This study aimed to verify the effectiveness of *O. vulgare* L. essential oil to inhibit the growth/survival of various food spoiling yeasts. Anti-yeast activity was studied by determining the MIC by solid medium diffusion and microplate bioassay, as well as observing the effect of the essential oil MIC on the yeast cell viability. *O. vulgare* essential oil showed effectiveness to inhibit the growth of all assayed yeasts with MIC values for the most ones of 20 and 0.6 μL/mL when determined, respectively, by solid medium diffusion and microplate bioassay. Solid medium diffusion MIC presented statistically significant inhibitory effects ($P < 0.05$) on yeast cell viability, mainly when interacting with *Candida albicans* and *Candida krusei*. On the other hand, the microplate MIC just provided statistically significant inhibitory effects on the cell viability when interacting with *C. krusei*. These data show the anti-yeast property of *O. vulgare* essential oil.

Keywords: *Origanum vulgare* L.; Essential oil; Yeasts; Foods

1. Introduction

Yeasts are widely distributed in nature and are able to spoil many foods such as wines, cheese, vinegar, beverages, juices, fruits, salads, sugar and meat, causing changes in odor, color, taste and texture (Ray, 1996). *Candida*, *Pichia*, *Rhodotorula*, *Torulopsis*, *Saccharomyces*, *Zygossacharomyces*, *Hansenula* and *Trichosporon* are some important food spoiling yeasts (Forsythe, 2004; Wojtatowicz, Chrzanowska, Juskekyk, Skib, & Gdula, 2002). Microbial spoilage has been an important factor influencing both the cost and food availability (Graham, 1980; Riedel, 2005; Walker, 1988).

Consumers have demanded more natural foods, with low levels of chemical additives and less processed, however still possessing a long shelf-life. Also, food legislation has restricted the use of some synthetic antimicrobials based on a possible toxicity for consumers (Brul & Coote, 1999; Burt, 2004). In this panorama, spices have emerged as effective compounds to provide microbiological safety of foods. Spices are rich in essential oils, which are composed of many compounds (eugenol, citral, pinene, thymol, cinnamic acid, carvacrol) characterized by a prominent antimicrobial activity (Juglal, Govinden, & Odhav, 2002; Konning, Agyare, & Enninson, 2004; Marino, Bersani, & Comi, 2001; Ngane, Biyiti, Bouchet, Nkengfack, & Zollo, 2003). From antiquity, in addition to spices and their derivatives being used for flavoring foods and beverages and for medication, they have been highly valued for their use as antimicrobials (Baydar, Sagdiç, Ozkan, & Karadogan, 2004; Ozcan, 1998).

Origanum vulgare L., Lamiaceae family, is widely known as possessing therapeutic properties (diaphoretic, carminative, antispasmodic, antiseptic, tonic) being used in traditional medicine systems in many countries (Sagdiç, Kusçu, Ozkan, & Ozçelik, 2002; Sahin et al., 2004). It has been

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widely used in agricultural, pharmaceutical and cosmetic industries as a culinary herb, flavoring substances in food products, alcoholic beverages and perfumery for its spicy fragrance (Aligianis, Kalpoutzakis, Mitaku, & Chinou, 2001; Dorman & Deans, 2000; Novak et al., 2000). Although, some researchers have found antimicrobial activity in *O. vulgare* L. (Baydar et al., 2004; Chun, Vattern, Lin, & Shetty, 2004; Nostro et al., 2004; Skandamis, Tsigarida, & Nychas, 2002), there is a lack of information about their inhibitory effect on the growth of food spoiling yeasts. The aim of this study was to evaluate the effectiveness of *O. vulgare* L. essential oil for inhibiting the growth/survival of some yeasts recognized as potential food spoiling microorganisms.

2. Material and methods

2.1. Essential oil

O. vulgare L. essential oil was obtained from Ferquima Ind. e Com. Ltd. (Vargem Grande Paulista, São Paulo, Brazil) and its quality parameters (appearance, color, purity, odor, density—20 °C, refraction index—20 °C) were described in a accompanying technical report. This provider produces and commercializes essential oils on an industrial scale. The essential oil was assayed at concentrations of 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.62 and 0.31 μL/mL, the solutions being prepared according to Souza, Lima, Freire, and Sousa (2005a).

2.2. Yeasts strains

Candida albicans ATCC 7645, *C. krusei* ATCC 6258, *C. tropicalis* MD 37, *Pichia minuscula* NI 7638, *P. ohmeri* ATCC 46053, *Rhodotorula rubra* LBFHC 1096 and *Saccharomyces cerevisae* ATCC 2601 strains were used as test microorganisms. The strains were supplied by National Institute of Quality in Health, FIOCRUZ, Rio de Janeiro, Brazil and Laboratory of Mycology, Pharmaceutical Sciences Department, Health Sciences Center, Federal University of Paraíba, Brazil. Stock cultures were maintained on Sabouraud agar (Accumedia) slants at 4 °C. Inocula were obtained from overnight cultures on Sabouraud agar slants at 28–30 °C and diluted in sterile PBS to a final concentration of 10^6 CFU/mL (adjusted according to the turbidity of 0.5 McFarland scale tube).

2.3. Antimicrobial assays

2.3.1. Solid medium diffusion method

Solid medium diffusion procedure using wells in dishes was used to determine the anti-yeast activity of *O. vulgare* essential oil. For this, 1 mL of the yeast suspension was uniformly spread on sterile Sabouraud agar petri dishes. After inoculum absorption by agar, wells were made using sterile glass tubes (diameter 6 mm) which were filled with 50 μL of the essential oil solutions (160–1.25 μL/mL) (Hadacek & Greger, 2000; Souza et al., 2005a). The system was incubated at 28–30 °C/48 h. At the end of the incubation period, the MIC was the lowest essential oil concentration showing growth inhibition halos with diameters equal to or greater than 10 mm. Controls included in this assay were essential oil replaced by sterile water.

2.3.2. Microplate bioassay

In addition to the solid medium diffusion procedure, the microplate bioassay (microdilution) was used to study the anti-yeast activity of *O. vulgare* essential oil. The 96-well plates were prepared by dispensing into each well 100 μL of double strength Sabouraud broth (Accumedia) inoculated with the yeast inoculum prior to the assay. An aliquot (100 μL) of the essential oil solutions at their respective concentrations was transferred into seven consecutive wells. The final volume in each well was 200 μL. The solution having the highest concentration (10 μL/mL) was added into the first well, so that the smallest concentration (0.31 μL/mL) was added into the penultimate well. The last well, containing 200 μL of Sabouraud broth inoculated with the yeast inoculum, was used as the positive control. The microplate was aseptically sealed, followed by mixing on a plate shaker (300 rpm) for 30 s, and incubated at 28–30 °C/48 h (Sahin et al., 2004; Viljoen et al., 2003). The MIC was defined as the lowest concentration of the essential oil able to provide visible yeast growth inhibition after the end of the incubation time (Cellini, Di Campili, Masulli, Di Bartolomeo, & Allocti, 1996; Espinell Infroff, 1992).

2.3.3. Kill time study

Kill time assay was carried out with the MIC values found in the solid medium diffusion procedure and microplate bioassay, using the viable cells count method. 5 mL of double strength Sabouraud broth was inoculated with 1 mL of the yeast suspension. After that, 4 mL of *O. vulgare* essential oil solution, with concentration adjusted to provide an essential oil final concentration similar to the MIC previously determined, was added to the system and followed for shaking using vortex for 30 s. The system was incubated at 28–30 °C. At different time intervals (1, 2, 4, 8, 12 and 24 h) post-incubation, 1 mL of the suspension was serially diluted in PBS (10^{-1}–10^{-5}) and spread on Sabouraud agar petri dishes and incubated for 28–30 °C/48 h (Arora & Kaur, 1999; Viljoen et al., 2003). The mean number of colonies (CFU/mL) was counted and compared with that found in the control assay in which the essential oil solution was replaced by sterile distilled water. The assay was repeated three times and the results were expressed in log of CFU/mL.

2.4. Statistical analysis

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

3. Results and discussion

The current necessity of discovering new antimicrobial compounds in all fields of microbial control has stimulated research regarding the antimicrobial properties of plant compounds (Ristori, Pereira, & Gelli, 2002; Utama, Wills, Ben-Yehoshua, & Kuesk, 2001). In this renewed interest, *O. vulgare* L. and derivatives have presented prominent results as antimicrobial agents to be applied in food bioconservation systems (Daferera, Ziogas, & Polissiou, 2003; Souza, Stamford, Lima, Trajano, & Barbosa-Filho, 2005b). Table 1 shows the MIC of *O. vulgare* essential oil on food spoiling yeasts determined by solid medium diffusion technique. The results showed that the essential oil had a substantial inhibitory effect on all assayed yeast strains noted by large growth inhibition halos. Most assayed strains showed an MIC of 10 μL/mL. The highest inhibitory activity was against *P. minuscula* which showed the lowest MIC (5 μL/mL) and the largest growth inhibition halos. On the other hand, *S. cerevisae* and *K. krusei* were the least sensitive yeasts with an MIC of 20 μL/mL, however *S. cerevisae* showed the smallest growth inhibition halo diameters when compared to all other strains. This high antimicrobial activity of *O. vulgare* essential oil supports the results found by other researchers (Chun et al., 2004; Marino et al., 2001; Skandamis et al., 2002).

Table 2 shows the MIC of *O. vulgare* essential oil on food spoiling yeasts determined by microplate bioassay. As can be seen, the MIC values found for microplate assay was always lower that those found in the solid medium diffusion assay. The MIC varied between 1.25 and 0.62 μL/mL. In general, the assayed strains presented similar behaviors in the two methods used to determine the MIC. The strains more or less sensitive to *O. vulgare* essential oil in the solid medium diffusion assay, showed the same behavior in the microplate assay, excepting *R. rubra*. This correlation between these two methods was also described by Viljoen et al. (2003).

Aligianis et al. (2001) proposed a classification for the antimicrobial activity of plant products, based on the MIC results as follows: strong inhibitors—MIC up to 0.5 μL/mL; moderate inhibitors—MIC between 0.6 and 1.5 μL/mL; weak inhibitors—MIC above 1.6 μL/mL. Thus, based on the MIC results found in microplate assays the *O. vulgare* essential oil presented an interesting potential as anti-yeast compound, since most yeast strains showed an MIC of 0.6 μL/mL.

Some researches regarding the antimicrobial properties of essential oils have found different results of MIC when using different methods for its determination, so that the microplate bioassay has always shown the lowest MIC values (Annuck et al., 1999; Burt & Reinders, 2003; Duarte, Figueira, Sartoratto, Garcia, & Delarmelina, 2005; Mann & Markhan, 1998; Sahin et al., 2004). Different results could be related mainly to the variation of the neat essential oil on the disc or into the well, disc or well size, agar composition as well as the volatility of the essential oil in an open air system when using the solid medium diffusion technique (Pattnail, Subramanyan, & Kole, 1996; Pauli & Kubezka, 1997; Viljoen et al., 2003). Inouye, Takizawa, Uchida, and Yamaguchi (2001) found MIC values two-to-eightfold lower when preventing the essential oil evaporation by sealing the plates.

The effect of *O. vulgare* essential oil MIC on the viability of food spoiling yeasts is shown in Figs. 1–3. The essential oil decreased the growth rate for all strains. However, the most intense inhibitory effect on cell viability was found to be the MIC as determined by the solid medium diffusion method, showing statistically significant differences

Table 1
Origanum vulgare L. essential oil MIC on food spoiling yeasts determined by solid medium diffusion[a]

Yeasts	*Origanum vulgare* L. essential oil[b]							
	160	80	40	20	10	5	2.5	1.25
C. albicans	38	28	20	12	10	0	0	0
C. krusei	32	25	15	12	0	0	0	0
C. tropicalis	35	27	21	14	11	0	0	0
P. minuscula	39	36	31	21	16	11	0	0
P. ohmeri	33	28	16	13	10	0	0	0
R. rubra	38	34	30	28	14	0	0	0
S. cerevisae	26	22	14	11	0	0	0	0

[a] Results expressed in millimeters of yeast growth inhibition halos.
[b] μL/mL.

Table 2
Origanum vulgare L. essential oil MIC on food spoiling yeasts determined by microplate bioassay[a]

Yeasts	*Origanum vulgare* L. essential oil					
	10	5	2.5	1.25	0.62	0.31
C. albicans	+	+	+	+	+	–
C. krusei	+	+	+	+	–	–
C. tropicalis	+	+	+	+	+	–
P. minuscula	+	+	+	+	+	–
P. ohmeri	+	+	+	+	+	–
R. rubra	+	+	+	+	–	–
S. cerevisae	+	+	+	+	–	–

(+): yeast growth inhibition; (–): no yeast growth inhibition.
[a] μL/mL.

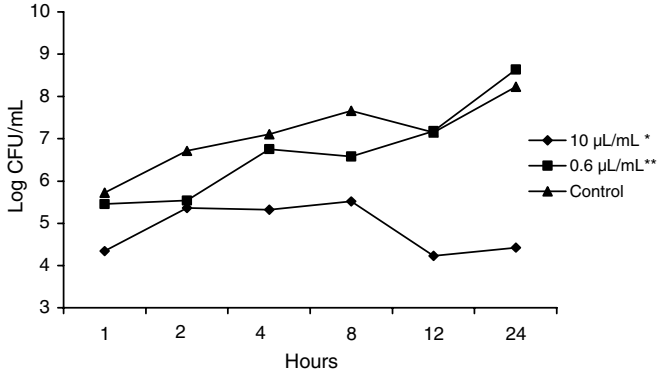

Fig. 1. Effect of *Origanum vulgare* L. essential oil MIC on the *C. albicans* viable cells number (★ solid medium diffusion MIC, ★★ microplate MIC).

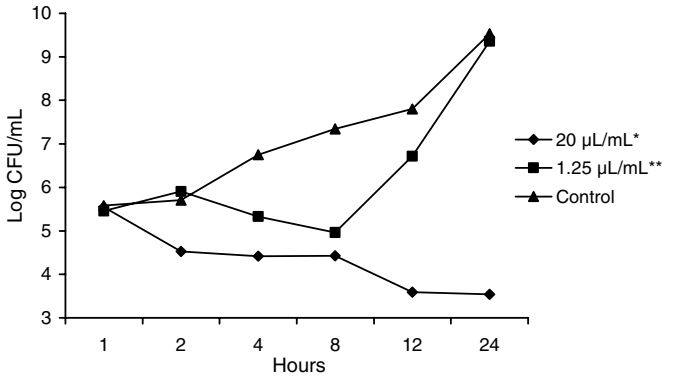

Fig. 2. Effect of *Origanum vulgare* L. essential oil MIC on the *C. krusei* viable cells number (* solid medium diffusion MIC, ** microplate MIC).

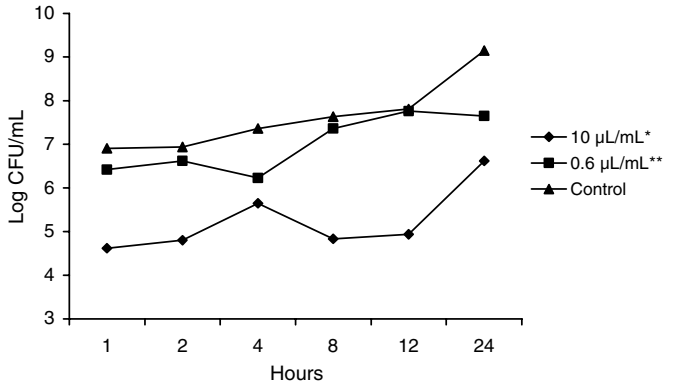

Fig. 3. Effect of *Origanum vulgare* L. essential oil MIC on the *C. tropicalis* viable cells number (* solid medium diffusion MIC, ** microplate MIC).

($P < 0.05$) with respect to the control treatment. Microplate MICs caused a statistically significant difference ($P < 0.05$) only when interacting with *C. krusei* (Fig. 2). Diffusion technique MIC provided a static activity on *C. tropicalis* (Fig. 3) and this property was maintained for at least 12 h. On the other hand, was observed a prominent cidal effect on *C. albicans* (Fig. 1) and *C. krusei* (Fig. 2) during 24 h of exposure. A compound has been considered as having a strong fungicidal effect when is able to cause a decrease of 99.9% (3 log cycles) of the initial inoculum (Espinell Infroff, 1992).

Some studies have found a strong cidal effect (total elimination of the microbial initial inoculum) of extracts and *O. vulgare* hydrosols after 3 days of interaction (Sagdiç et al., 2002; Sagdiç, 2003). *O. vulgare* essential oil is rich in phenolic compounds, which are believed to be responsible for its prominent antimicrobial activity (Marino et al., 2001). Carvacrol, γ-terpinene, α-terpinene, thymol, *p*-cimene, γ-terpineol, sabinene, myrcene, caryophyllene, germancrene, spathulenol are some chemical compounds found in *O. vulgare* essential oil (Daferera et al., 2003; Sahin et al., 2004; Velluti, Sanchis, Ramos, & Egido, 2003). Phenolic compounds are capable of dissolving in the microbial membrane, thus penetrating inside the cell, where they interact with cellular metabolic mechanisms (Baydar et al., 2004; Chun et al., 2004). Cytoplasm membrane disturbance, rupture of proton motive force and cytoplasm content coagulation are some mechanisms involved in the antimicrobial properties of essential oils (Carson, Mee, & RiIey, 2002; Sikkema, De Bont, & Poolman, 1995).

Our data confirm the antimicrobial potential of spice essential oils, particularly the anti-yeast activity of *O. vulgare* essential oil. In addition, our results support the possibility of the use of *O. vulgare* derivatives as potential alternative antimicrobial compounds to be applied in food conservation, since this essential oil is considered toxicologically safe. However, further research is needed to verify its antimicrobial effectiveness in food matrices, as well as to evaluate its effectiveness to protect foods against pathogen and spoiling microorganisms throughout shelf-life.

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