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Influence of emulsifier type on the antifungal activity of cinnamon leaf, lemon and bergamot oil nanoemulsions against *Aspergillus niger*

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ABSTRACT

Only exiguous data are currently available on the antifungal properties of essential oil (EO) nanoemulsions against spore-forming microorganisms. The aim of this work is to develop physically stable nanoemulsion-based delivery systems for different EOs (cinnamon leaf, lemon, and bergamot), to exploit their antifungal properties against *Aspergillus niger*. The inhibition of mycelial radial growth and spore germination were used as indicators of antifungal activity of the nanoemulsions, which were prepared at 3 wt% EO, using non-ionic Tween 80 (T80) or anionic whey protein isolate (WPI) (1 wt%) as emulsifiers, and sunflower oil (1 wt%) as ripening inhibitor. The nanoemulsions were physically stable over seven days of accelerated aging at 35 °C.

The minimal inhibitory concentration of free cinnamon leaf and of both citrus EOs were 0.35 and 5.50 μg/g, respectively. The encapsulation of cinnamon leaf EO in nanoemulsions significantly enhanced the inhibiting effect against *A. niger* mycelial growth and spore germination, with respect to the free EO. In contrast, for citrus EOs, the encapsulation in nanoemulsions generally decreased the antifungal activity, likely because of the nanoemulsion acting as a hydrophobic sink for the main constituents of citrus EOs. The emulsifier played a fundamental role in the resulting antifungal activity, with WPI-based nanoemulsions being more effective in inhibiting the mycelial growth and the spore germination of *A. niger* than T80-based ones. The antifungal action was correlated to the morphological alterations observed in *A. niger*, such as the loss of cytoplasm in fungal hyphae and hyphal tip. The results of this study show the importance of nanoemulsions design in the development of efficient and stable natural antifungal agents for food applications.

1. Introduction

Food product deterioration during storage, caused by fungi, and especially by moulds, is responsible for significant economic losses to the food industry. The most common mould genus is *Aspergillus*, which is a plant, animal, and human pathogen. It can contaminate agricultural products at different stages such as pre-harvesting, harvesting, processing and handling. The changes associated with spoilage by *Aspergillus* species encompass the sensorial, nutritional and qualitative product properties. However, the most notable consequence of their presence is the contamination of food and feeds with mycotoxins (Perrone et al., 2007).

The use of chemical preservatives as antifungal agents to control fungal spoilage had become a common practice in the last decades. However, following the recent increasing consumers' trends towards more natural and healthy food products, food processors have started to search for safer alternatives to replace synthetic additives. Plant products have been recognized and employed for food protection since many years (Rodriguez-Lafuente, Nerin de la Puerta, & Batlle, 2009). Essential oils (EOs) belong to one of the most promising classes of natural antifungal preservatives (Tian et al., 2011; Varma & Dubey, 2001). However, despite their enormous potential of application, the use of EOs as food preservatives is strongly limited by their high volatility, low water-solubility and

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strong susceptibility to environmental conditions. The encapsulation of EOs in oil-in-water (O/W) nanoemulsions significantly improves their water dispersibility, and, by providing large surface areas of contact with the microorganisms, also contributes to enhancing the antimicrobial effectiveness of EOs (Donsì & Ferrari, 2016).

The main purpose of this study is the investigation of the effects on the antifungal activity against *Aspergillus niger* of three different EOs (cinnamon leaf, lemon, and bergamot), when encapsulated in O/W nanoemulsions, formulated with two different emulsifiers of approved use in foods, such as T80 and WPI. In particular, the antifungal activity of the developed nanoemulsions is evaluated in terms of the induced inhibition of fungal growth, of mycelial growth, and of spore germination, as well as of the morphological damages caused to fungal hyphae and hyphal tips. The T80, a non-ionic low-mass surfactant, is characterized by high surface activity, which translates in the quick absorption at O/W interfaces, efficiently preventing the coalescence of the droplets (Li et al., 2015). Negatively charged whey proteins are instead able to form a protective membrane around the oil droplets, which prevents droplet aggregation by steric hindrance and electrostatic repulsion (Hesbishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015; Teo et al., 2016).

2. Material and methods

2.1. Microorganism, culture media, and reagents

The strain of *Aspergillus niger* (CECT 20156) was supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). For culture media, Potato Dextrose Broth and Potato Dextrose Agar were used, all provided by Scharlab (Barcelona, Spain).

The EOs used in this work were cinnamon leaf (CEO), purchased from Sigma-Aldrich (Milan, Italy), lemon (LEO) and bergamot (BEO), which were both supplied by CAPUA s.r.l. (Reggio Calabria, Italy). In some emulsion formulations, the EOs were mixed with sunflower oil purchased from Sagra (Lucca, Italy). T80 (Sigma-Aldrich, Milan, Italy) and WPI (Volactive UltraWhey 90, Volac Socoor S.r.l., Italy) were employed as emulsifying agents. According to manufacturer specifications, T80 has a molecular weight of 1.31 kDa, whereas WPI, consisting of β-lactoglobulin (50–60% w/w), glycomacropeptide (15–20% w/w), α-lactalbumin (15–20% w/w), bovine serum albumin (1.0–2.0% w/w), immunoglobulin G (1.0–2.0% w/w), immunoglobulin A (0.1–1.0% w/w), and lactoferrin (0.1–0.5% w/w), had an average molecular weight of 18.2 kDa.

A. niger mycelial material and spores were fixed with a lactophenol-cotton blue solution, purchased from Sigma-Aldrich (Milan, Italy).

2.2. Characterization of free EOs

2.2.1. Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of CEO, LEO, and BEO was performed on a 6890/5975 inert GC-MS Agilent Technologies, USA, equipped with a HP-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm) to determine the EOs composition. The oven temperature was held at 60 °C for 3 min, then raised to 100 °C at 10°C/min, to 140 °C at 5°C/min, and finally to 240 °C at 20°C/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 250 °C and 230 °C, respectively. Parameters for MS analysis were EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40–550 *amu*. The identification of the EO components was performed by matching mass spectra with the standard mass spectra from the NIST MS Search 2.0 library. The results were expressed as the percentage of relative area (%) of two runs for each EO.

2.2.2. Antifungal activity of free EOs

2.2.2.1. Mycelial growth and minimal inhibitory concentration assay. The CEO, LEO, and BEO were individually examined against *A. niger* as described by Ribes, Fuentes, Talens, and Barat (2016). The mould was inoculated on Potato Dextrose Agar and incubated at 25 °C for 7 days. Subsequently, the spores were counted in a haemocytometer to achieve an inoculum density of 10^6 CFU/mL.

Different EO concentrations were examined on the basis of previous studies (Gemeda, Woldeamanuel, Asrat, & Debella, 2014; Sharma & Tripathi, 2008): 0.10, 0.25, 0.35, 0.50, 1.00, 1.50 and 2.00 μg/g for CEO; and 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50 and 6.00 μg/g for LEO and BEO. The selected maximum concentrations are all below the solubilities in water of the main components, which are of 1.9 mol/mol for eugenol and of 1.0 mol/mol for D-limonene (Miller & Hawthorne, 2000), corresponding to 17.3 μg/g and 7.6 μg/g, respectively. The EOs were added to aliquots of 15 g of Potato Dextrose Agar containing 0.1 wt% T80, to ensure the even dispersion of the EOs, according to a procedure previously described (Ribes et al., 2016; Tao, Jia, & Zhou, 2014), into Petri dishes. The EOs were added to the culture medium at 50 °C. Control Petri dishes, without EOs, were prepared following the same procedure. The centre of each plate tested was inoculated with a Potato Dextrose Agar disc (7 mm diameter) taken from the edge of zero-day-old fungi culture, previously spread with 100 μL of the spore solution (10^6 CFU/mL). Each plate was sealed with Parafilm® and incubated for 7 days at 25 °C.

At the end of the incubation period, the edge of the fungi culture was observed using a light microscope at 100×, 200× and 400× magnification. The mycelial material was fixed by using lactophenol-cotton blue solution. Growth inhibition of treatment against control was calculated using Equation (1):

$$\textit{Mycelial growth inhibition}(\%) = [C - T/C] \times 100 \quad (1)$$

where *C* and *T* represented the mycelial growth (mm) in the control and treated plates, respectively.

Furthermore, the Minimal Inhibitory Concentration (MIC) of the CEO, LEO, and BEO against *A. niger* was determined. The MIC was defined as the lowest concentration in the serial dilution of the antifungal agents, which resulted in the lack of visible growth after 7 days of incubation at 25 °C.

All tests were performed in duplicate, for each treatment, which was repeated twice.

2.2.2.2. Spore germination assay. Spores from seven-day-old *A. niger* culture were collected by adding 1 mL of sterile water containing 0.1 wt% T80 to each Petri plate and rubbing the surface with a sterile L-shaped spreader. The spores were transferred to a tube containing 5 mL of sterile water and 0.1 wt% T80 and counted in a haemocytometer to achieve an inoculum density of 10^6 CFU/mL.

The same EO concentrations used in the mycelial growth assay were examined. In this case, they were added to 5 mL of Potato Dextrose Broth with 0.1 wt% of T80, to ensure the even dispersion of the EOs, and 100 μL of the inoculum density were added to each tube. Potato Dextrose Broth tubes with no EOs were used as controls. The tubes were incubated 24 h at 25 °C. At the end of the incubation period, germinated spores were observed using a light microscope at 400× magnification. Each slide was fixed in lactophenol-cotton blue solution. The assay was run in duplicate and the efficacy of the EO treatments was evaluated by looking for the presence of germ tubes. Each treatment was repeated twice.