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Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food

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A B S T R A C T

The antifungal activities of eight essential oils (EOs) namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme were evaluated for their ability to inhibit growth of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium chrysogenum*. The antifungal activity of the EOs was assessed by the minimum inhibitory concentration (MIC) using 96-well microplate analysis. The interactions between different EO combinations were done by the checkerboard technique. The highest antifungal activity was exhibited by oregano and thyme which showed lower MIC values amongst all the tested fungi. The antifungal activity of the other EOs could be appropriately ranked in a descending sequence of cinnamon, peppermint, tea tree and basil. Eucalyptus and mandarin showed the least efficiency as they could not inhibit any of the fungal growth at 10,000 ppm. The interaction between these two EOs also showed no interaction on the tested species. A combined formulation of oregano and thyme resulted in a synergistic effect, showing enhanced efficiency against *A. flavus* and *A. parasiticus* and *P. chrysogenum*. Mixtures of peppermint and tea tree produced synergistic effect against *A. niger*. Application of a modified Gompertz model considering fungal growth parameters like maximum colony diameter, maximum growth rate and lag time periods, under the various EO treatment scenarios, showed that the model could adequately describe and predict the growth of the tested fungi under these conditions.

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1. Introduction

Fungi are regarded as one of the main concerns in food storage (Zhaveh et al., 2015). *Aspergillus*, *Penicillium* and *Fusarium* are the main fungi associated with wheat, rye and corn grains under field and storage conditions (Krisch et al., 2011). Fungal growth on raw and processed foods may result in several kinds of spoilage and include textural and sensory changes, off-flavor development and odour emission, rotting and formation of pathogenic and allergenic propagules (Dellavalle et al., 2011). The deterioration of sensorial properties is often due to the production of exoenzymes during fungal growth. Once inside the food, these enzymes may continue their activities independent of the destruction or removal of the mycelium. In addition, the production of mycotoxins by fungi, in stored food commodities constitutes a serious health threat to humans and livestock. Five types of mycotoxins are deemed noxious world-wide for human health: aflatoxins, ochratoxin A, fumonisins, certain trichothecenes and zearalenone (Pitt et al., 2000). Long-term ingestion of these toxins as a result of eating contaminated foods has been associated with liver and kidney tumors in animals and humans. Some mycotoxins can cause autoimmune illnesses, while some are teratogenic, carcinogenic and mutagenic (Angelini et al., 2006; Garcia et al., 2009; Krisch et al., 2011). Furthermore, these toxins can accelerate lipid oxidation due to the chain reaction of free radical oxidation. Certain mycotoxins have been reported to produce free radicals which certainly impose an undesirable influence on human health (Alves-Silva et al., 2013). Hence, the development of multiple measures to prevent fungal growth, mycotoxin production and free radical generation has become a crucial aspect to combat food losses and preserve food quality during storage and transport.

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The food industry has tended to reduce the use of chemical preservatives of antifungal activity due the pressure imposed by consumers and legal authorities to either completely remove these toxic compounds or to adopt more natural alternatives for the maintenance or extension of product shelf life (Beyki et al., 2014). Essential oils (EOs) represent one of these natural additives and bear potent biological activities. In recent years, numerous *in vitro* and *in vivo* studies have reported the antifungal effects of plant EOs to control food spoilage (Gibriel et al., 2011; Mohammadi and Aminifard, 2013; Sumalan et al., 2013; Tian et al., 2012). However, the biological activity of EOs varies greatly with individual EO, depending on the chemical composition which is specific to plant parts used, method of extraction, harvesting season etc (Chaubey, 2007; Vitoratos et al., 2013).

The aim of the current research was to evaluate the inhibition activity of eight EOs against *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium chrysogenum* by determining (i) the minimum inhibitory concentration (MIC) of the EOs and (ii) the possible synergistic effects between EO combinations, and finally (iii) antifungal activities of the volatile components resulting from the EOs to better understand the inhibitory kinetics caused by EOs on fungal growth.

2. Materials and methods

2.1. Preparation of essential oil emulsion

Basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme EOs were obtained from Robert & Fils (Ghislenghien, Belgium) and stored at 4 °C prior to use. Each EO was prepared as an emulsion containing 2.5% (v/v) of EO and 2.5% (v/v) of Tween 20 (Laboratoire Mat, QC, Canada). The mixtures were homogenized for 5 min with an Ultra-Turrax homogenizator (model TP18/1059, Germany) at 20,000 rpm to obtain a colloidal suspension. The emulsions were aseptically filtered using a 0.45 μm pore size sterile filter. The mixtures were then stored at 4 °C. The major component (provided by the manufacturer) of these EOs are presented in Table 1.

2.2. Fungal inocula and assay media

A. niger (ATCC 1015), *A. flavus* (ATCC 9643), *A. parasiticus* (ATCC 16869), and *P. chrysogenum* (ATCC 10106) were used for the assays. Each fungal species was grown and maintained in potato dextrose broth (PDB, Difco, Becton Dickinson) containing glycerol (10% v/v). Prior to each experiment, stock cultures were propagated through two consecutive 48 h growth cycles in PDB medium at 28 °C ± 2 °C. The cultures were pre-cultured in PDA for 3 days at 28 °C ± 2 °C. Conidia were isolated from the agar media using sterile saline containing 0.05% Tween 80, and the filtrate was adjusted to 1×10^7 conidia/mL for broth dilution and checkerboard method and 1×10^8 conidia/mL for vapor contact assay by using a microscope (Inouye et al., 2006).

Table 1
List of EOs and their major active components.

Essential oil	Latin name	Major components
Basil	*Ocimum basilicum*	Estragole, Eugenol, linalool
Cinnamon	*Cinnamomum zeylandicum*	Linalool, trans-cinnamaldehyde
Eucalyptus	*Eucalyptus globulus*	1,8 cineole
Mandarin	*Citrus reticulata*	Limonene, γ terpinene
Oregano	*Origanum vulgare*	Carvacrol, thymol
Peppermint	*Mentha piperita*	Menthol, menthone,
Tea tree	*Melaleuca alternifolia*	Terpineol
Thyme	*Thymus vulgaris*	Thymol, carvacrol, γ-terpinene

2.3. Determination of the minimum inhibitory concentrations (MIC) using broth dilution method

The method for determination of MIC was adopted from Turgis et al. (2012) with slight modification. All EOs were diluted in Potato Dextrose Broth (PDB) medium to obtain serial concentrations of 10,000–10 ppm. A sample of 125 μL of the serially diluted EOs was pipetted into 96 wells microplate (Sarstedt, QC, Canada). Each sample well was inoculated with 15 μl of fungi at a concentration of 10^7 CFU/mL in order to obtain 1×10^6 conidia/mL of final concentration. The microplate was incubated aerobically for 36 h at 28 °C. After incubation, the absorbance was measured at 595 nm using an Ultra Microplate Reader (Biotek instruments, VT, USA). Sterile PDB medium was incubated under the same condition and used as a negative control. PDB medium incubated with a specific fungal species (without EO) was used as a positive control of growth. The minimum inhibitory concentration (MIC) was determined as the lowest EO concentration showing a complete growth inhibition of the tested fungal strains. This was evaluated by measuring the absorbance that is equal to the absorbance of the blank (sterile PDB only).

2.4. Assessing interaction between EO mixtures by the checkerboard method

Combination assays were evaluated based on a checkerboard procedure described by Turgis et al. (2012). The checkerboard method was performed to obtain the fractional inhibitory concentration (FIC) index of mixtures of EO combinations against each mold species. The index was calculated by adding the FIC values of EO (a) denoted by FIC_a and (b) denoted by FIC_b. The FIC_a and FIC_b values represented the fraction of the lowest concentrations of EOs and mixtures of EOs, respectively, that caused inhibition of fungal growth in the combination tests. The calculations were performed using the following equations

$$FIC_a = MIC_{a,combined} / MIC_{a,alone} \quad (1)$$

$$FIC_b = MIC_{b,combined} / MIC_{b,alone} \quad (2)$$

$$FIC = FIC_a + FIC_b \quad (3)$$

Based on the above, the FIC of an EO could be equated to the concentration which caused deactivation of the fungal species when used in combination with another EO divided by the concentration that had the same effect when used alone (Gutierrez et al., 2009; Mamoudou et al., 2010). An $FIC \leq 0.5$ was interpreted as a synergistic effect, $0.5 \leq FIC \leq 1$ represented as an additive effect, $FIC \leq 4$ represented as no interactive effect and $FIC > 4$ indicated an antagonistic effect between two tested EOs (Gutierrez et al., 2008; Krisch et al., 2011).

2.5. Vapor contact assays

Vapor contact assays were performed based on the method described by Inouye et al. (2006). A sample of 1 mL containing 1×10^8 of conidial suspension of the each fungal species were added to 100 mL of agar medium containing 1% peptone, 1% glucose and 1% agarose at 50 °C. A volume of 3 mL of the prepared mixture was overlaid onto the surface of hardened PDA medium (20 mL) in a Petri dish (83 mm in diameter) to prepare a double layered agar medium. Sterile filter paper (10 mm diameter) was placed at the

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