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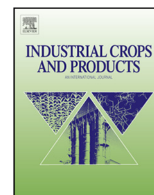

Antifungal activity of selected essential oils against fungi isolated from medicinal plant

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ABSTRACT

The development of protective products with natural origin as alternatives to synthetic fungicides is currently in the spotlight. Qualitative and quantitative chemical analysis of 16 selected essential oils was determined. Then, antifungal properties determined by *in vitro* microdilution method against 21 fungi isolated from herbal drugs were evaluated. All tested oils showed some antifungal activity against all fungi used. Savory, thyme and oregano oils, characterized by the presence of phenol such as carvacrol and thymol, and rose oil containing mainly monoterpene alcohols (citronellol and geraniol) proved to be the most effective inhibitor of all fungi tested. Also, combination of particular oils showed reduction of the MIC values when combined, commendatory mixtures for potential application in practice. Moreover, the reduction of the total number of fungi, *in situ*, using selected essential oils was determined.

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1. Introduction

Interest in traditional medicine and, in particular, herbal medicines, has increased substantially in both developed and developing countries over the past two decades. Global and national markets for medicinal herbs have been growing rapidly and significant economic gains are being realized. As a consequence, the safety and quality of herbal medicines have become increasingly important concerns for health authorities and the public alike. The safety and quality of raw medicinal plant materials and finished products depend on factors that may be classified as intrinsic (genetic) or extrinsic (environment, collection methods, cultivation, harvest, post-harvest processing, transport and storage practices). Inadvertent contamination by microbial or chemical agents during any of the production stages can also lead to deterioration in safety and quality. In addition to the crop loses, presence of the fungi in medicinal plants reduces their quality and usefulness (Essono et al., 2007). Moreover, under certain conditions, some of the ubiquitous fungal contaminants could secrete the toxic metabolites, mycotoxins, with powerful mutagenic and carcinogenic effect. Mycotoxins are thermo stable and cannot be destroyed by cooking. They have cumulative ability and are eliminated hard from the organism (Hashem and Alamri, 2010).

The usual antimicrobial chemicals used in agriculture for plant disease control (benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors) are associated with series of problems. Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity (Skandamis et al., 2001). For these reasons, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensified. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies (Rapp, 2004). The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Schuenzel and Harrison, 2002).

In the past decade, due to concerns regarding safety of the synthetic antimicrobial agents, the particular interest has been focused on the potential applications of essential oils as alternative

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Table 1
Essential oils.

Essential oil	Plant origin	Specification
Orange	*Citrus aurantium amara*	P0104578
Bergamot	*Citrus aurantium bergamia*	P0112136
Lemon	*Citrus limon*	P0110499
Coriander	*Coriandrum sativum*	P0112145
Eucalyptus	*Eucalyptus globulus*	P0112145
Anise	*Illicium verum*	S0100154
Lavender	*Lavandula angustifolia*	P0123527
Chamomile (blue)	*Matricaria recutita*	P0115610
Tea tree	*Melaleuca alternifolia*	P0123084
Basil	*Ocimum basilicum*	P0118460
Geranium	*Pelargonium graveolens*	P0114231
Rose	*Rosa damascena*	P0100578
Savory	*Satureja hortensis*	P0118884
Thyme	*Thymus vulgaris*	P0123774
Viola	*Viola odorata*	P0105637
Oregano	*Origanum heracleoticum*	na

chemical control measures. They have a broad spectrum of antifungal properties (Kalemba and Kunicka, 2003; Soković and van Griensven, 2006; Carmo et al., 2008; Koul et al., 2008) and they are environmentally friendly (biodegradable, do not leave toxic residues or by-products to contaminate the environment) (Abdel-Kader et al., 2011).

This study was undertaken to investigate the *in vitro* inhibitory effects of a number of essential oils, differing in chemical composition, against 21 phytopathogenic and saprophytic, pre-harvest and post-harvest, fungi isolated from medicinal herbs, antifungal activity of combinations of two oils *in vitro* as well as the *in situ* effect of selected oils.

2. Materials and methods

2.1. Essential oils

Essential oils used in this study are listed in Table 1. The oils were purchased from a Frey + Lau GmbH, Henstedt-Ulzburg, Germany, except for the essential oil of oregano, which was derived from the company Herba, Serbia.

2.2. Gas chromatography

Gas chromatography-flame ionization detector (GC-FID) and Gas chromatography–mass spectrometry (GC-MS) analysis was performed on an Agilent 7890A GC equipped with 5975 C inert XL EI/CI MSD and FID detector connected by capillary flow technology 2-way splitter with make-up. An HP-5MSI capillary column (30m × 0.25 mm × 0.25 μm) was used. The temperature for GC oven was programmed from 60 °C to 300 °C at 3 °C/min and hold for 10 min. Helium was used as carrier gas at 16.255 psi (constant pressure mode). The sample was analyzed in the splitless mode. The injection volume was 1 μL. GC detector temperature was 300 °C. MS data was acquired in EI mode, with scan range 30–550 *m*/*z*, source temperature was 230 °C and quadrupole temperature was 150 °C. Solvent delay was 3 min.

2.3. Fungi

Antifungal activity was tested using the twenty one fungi isolated from medicinal drugs and identified: *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata*, *Fusarium solani*, *Fusarium tricinctum*, *Fusarium sporotrichioides*, *Fusarium verticillioides*, *Fusarium oxysporum*, *Fusarium semitectum*, *Fusarium subglutinans*, *Fusarium equiseti*, *Penicillium* sp., *Chaetomium* sp., *Gliocladium roseum*, *Curvularia lunata*, *Verticillium dahliae*, *Trichoderma viride*, *Trichotechium roseum*, *Phomopsis* sp., *Phoma* sp. and *Myrothechium verrucaria*. As a source of isolation we used herbal drugs that showed the highest level of contamination with phytopathogenic and saprophytic fungi: mint (*Mentha piperita* L. folium et herb), nettle (*Urtica dioica* L. folium), marigold (*Calendula officinalis* L. flowers), horsetail (*Equisetum arvense* L. herb) and corn silk (*Zea mays* L. stigma) (Stević et al., 2012).

2.4. Antifungal assay in vitro

To investigate the antifungal activity of essential oil, a modified version of the microdilution technique was used (Hanel and Raether, 1988; Daouk et al., 1995). Fungal spores were washed from the surface of malt agar (MA) plates (in g/L: malt extract 50 and agar 15) with sterile 0,85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. Determination of MIC values was performed by a microdilution technique using 96-well microtiter plates. Tryptic Bile Soy Broth (TBS) (in g/L: casein 20, bile salts 1.5, X-B-D glucuronic acid 0.075 and dimethyl sulfoxide 3) was used as the basis in the well to which 0.01% Tween 80, different volumes of the investigated essential oils and fungal inoculum were added. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth were defined as the minimal concentrations which completely inhibited fungal growth (MIC). The minimal fungicidal concentrations (MFC) were determined by serial subcultivation of a 2 μL volume on microtiter plates containing 100 μL of broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum compared to fluconazole, used as a positive control.

2.5. Screening antifungal activity of combinations of two oils in vitro

The synergism, indifference, and antagonism of the combinations of two essential oils were screened on the selected eight fungal strains. MICs were transformed into fractional inhibitory concentration (FIC) to determine the interaction of two essential oils, in the following manner:

$$\text{FIC of A oil} = \frac{\text{MIC of A oil in presence of B oil}}{\text{MIC of A oil}}$$

$$\text{FIC of B oil} = \frac{\text{MIC of B oil in presence of A oil}}{\text{MIC of B oil}}$$

Fractional inhibitory concentration index (FIC index) for each oil was then calculated from FIC values as follows:

FIC index = FICA + FICB

where A was first oil and B was second oil in combinations.

The FIC index (FICi) was interpreted as: synergistic effect when FICi $\leq$ 0.5; indifferent effect when FICi was 0.5–2 and antagonistic effect when FICi $\geq$ 2 (da Silva et al., 2011).

Criteria for choosing oils for this assay was to combine two oils of different antifungal potential (high/low) and chemical composition and, on the other hand, two oils with similar antifungal potential (both high) and chemical composition. Also, target fungi for investigating combine activity of two oils were ones potential producers of mycotoxins and most frequently isolated from herbal drugs in our previous investigation (Stević et al., 2012).

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