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In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms

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ABSTRACT

The aim of this study was to evaluate the antimicrobial efficacy of selected plant essential oil (EO) combinations against four food-related microorganisms. Ten EOs were initially screened against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Saccharomyces cerevisiae* using agar disk diffusion and broth dilution methods. The highest efficacy against all the tested strains was shown when testing the oregano EO. EOs of basil and bergamot were active against the Gram-positive bacteria (*S. aureus* and *B. subtilis*), while perilla EO strongly inhibited the growth of yeast (*S. cerevisiae*). The chemical components of selected EOs were also analyzed by GC/MS. Phenols and terpenes were the major antimicrobial compounds in oregano and basil EOs. The dominant active components of bergamot EO were alcohols, esters and terpenes. For perilla EO, the major active constituents were mainly ketones. The checkerboard method was then used to investigate the antimicrobial efficacy of EO combinations by means of the fractional inhibitory concentration index (FICI). Based on an overall consideration of antimicrobial activity, organoleptic impact and cost, four EO combinations were selected and their MIC values were listed as follows: oregano–basil (0.313–0.313 μl/ml) for *E. coli*, basil–bergamot (0.313–0.156 μl/ml) for *S. aureus*, oregano–bergamot (0.313–0.313 μl/ml) for *B. subtilis* and oregano–perilla (0.313–0.156 μl/ml) for *S. cerevisiae*. Furthermore, the mechanisms of the antimicrobial action of EO combinations to the tested organisms were studied by the electronic microscopy observations of the cells and the measurement of the release of cell constituents. The electron micrographs of damaged cells and the significant increase of the cell constituents' release demonstrated that all EO combinations affected the cell membrane integrity.

1. Introduction

In spite of modern improvements in food production and preservation techniques, such as genetic engineering, irradiation of food, and modified-atmosphere packaging (WHO, 2002), food safety is a growing public health concern. The survival of microorganisms in food is an important problem, which can lead to spoilage and deteriorate the quality of food products (Celiktas et al., 2007) or cause infection and illness (Jacob, Mathiasen, & Powell, 2010). It is estimated that food-borne diarrheal diseases have already caused about 4 to 6 million deaths per year, with most of these occurring in young children (WHO, 2003). Although chemical preservative has been used for years, there is much controversial because they have been shown to cause respiratory or other health problems (Fleming-Jones & Smith, 2003). Therefore, it is necessary to find a novel way to reduce or eliminate food-related microorganisms during the shelf life of food products. In the meantime, the increasing demand of consumers for natural products has led to research on new antimicrobial agents from plants to improve the safety of products (Goni et al., 2009).

Essential oils (EOs) are the volatile oily liquids of the secondary metabolism of scented plants, which are obtained from different plant parts, such as flowers, leaves, seeds, bark, fruits and roots (Burt, 2004). Although some EOs have long been researched for their antibacterial, antifungal, antiviral, and antioxidant properties (Gao et al., 2011; Gilles, Zhao, An, & Agboola, 2010; Kordali et al., 2005; Mourey & Canillac, 2002; Prakash et al., 2011; Sylvestre, Pichette, Longtin, Nagau, & Legault, 2006), the recent enhancement of interest in 'green' consumerism has given rise to a renewal scientific awareness of them. It has been demonstrated that various medicine plants, spices and herbs containing EOs significantly inhibit a wide range of microorganisms. Moreover, different results in antimicrobial test were observed depending on measurement conditions, tested strains and the source of the antimicrobial compound (Turgis, Han, Caillet, & Lacroix, 2009). Considering their excellent antimicrobial function, EOs possess great potential as natural additives for food preservation.

Although desired antimicrobial activity of several EOs against pathogenic and spoilage microorganisms is performed in vitro test, it

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has generally been found that a higher concentration is needed to achieve the same effect in foods (Shelef, Jyothi, & Bulgarelli, 1984). This fact may lead to an organoleptic impact as the use of natural preservatives can alter the taste of food and exceed the flavor threshold acceptable to consumers (Hsieh, Mau, & Huang, 2001; Nazer, Kobilinsky, Tholozan, & Dubois-Brissonnet, 2005). Gutierrez, Rodriguez, Barry-Ryan, and Bourke (2008) found that lettuce samples treated with 500 or 1000 ppm concentration of thyme and lemon balm EOs were rejected because their bad effects on the organoleptic acceptability of foods.

A number of studies have focused on the synergistic activity of the EO in combination with antibiotic to minimize the side effects of the antibiotic (Mahboubi & Bidgoli, 2010; Rosato et al., 2009; Tohidpour, Sattari, Omidbaigi, Yadegar, & Nazemi, 2010), while the combinations of EO with other natural antibacterial compounds (e.g., nisin) was used in food to reduce the minimum effective dose of EO (Solomakos, Govaris, Koidis, & Botsoglou, 2008). However, a few studies regarding the synergistic effects of EO combinations to obtain effective antimicrobial activity at sufficiently low concentrations and consequently reduce negative sensory impact were performed (Gutierrez, Barry-Ryan, & Bourke, 2009). EOs in plants generally are mixtures of abundant components (Burt, 2004), therefore, the synergistic effects are perhaps more likely to achieve in EO combinations.

The present study aimed to: (1) screen the antimicrobial properties of ten plant EOs against four common food-related bacteria and yeasts, including *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Saccharomyces cerevisiae*; (2) determine the chemical composition of the selected EOs by GC/MS; and (3) access the efficacy of selected EO combinations against tested microorganisms to determine potential for their synergistic at low doses. The effects of selected EO combinations on the integrity of cell membranes were also studied in order to elucidate the antimicrobial mechanisms of action.

2. Material and methods

2.1. Antimicrobial agents

2.1.1. Essential oils

The commercial EOs used in this study were purchased from Guanxiang Chemicals Trading Co. Ltd. (Changsha, China), including patchouli EO (*Pogostemon cablin*), clary sage EO (*Salvia sclarea*), rosemary EO (*Rosmarinus officinalis*), basil EO (*Ocimum basilicum*), spearmint EO (*Mentha spicata*), oregano EO (*Origanum vulgare*), perilla EO (*Perilla arguta*), absinthe EO (*Artemisia absinthium*), bergamot EO (*Citrus bergamia*) and lavender EO (*Lavandula angustifolia*). The details of ten commercial plants EOs were listed in Table 1 and all EOs were stored at the temperature of 4–6 °C before analysis.

2.1.2. Antibiotic and chemical preservatives

The antibiotic selected in the study was kanamycin sulfate, which was purchased from Conba Pharmaceutical Co. Ltd. (Jinhua, Zhejiang, China). Three common chemical preservatives (sodium benzoate, potassium sorbate and methylparaben) were obtained from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China).

Table 1
The details of ten commercial plant essential oils.

Plant species	Common name	Distilled part
Pogostemon cablin	Patchouli	Leaf
Salvia sclarea	Clary sage	Leaf–flower
Rosmarinus officinalis	Rosemary	Branch
Ocimum basilicum	Basil	Leaf–flower
Mentha spicata	Spearmint	Stem–leaf
Origanum vulgare	Oregano	Whole plant
Perilla arguta	Perilla	Leaf
Artemisia absinthium	Absinthe	Leaf–branch
Citrus bergamia	Bergamot	Peel
Lavandula angustifolia	Lavender	Flower

2.2. Antimicrobial activity

2.2.1. Microbial strains and growth conditions

Four food-related microorganisms were used to assess the antimicrobial properties, including the Gram-positive *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, the Gram-negative *E. coli* ATCC 8739, and the yeast *S. cerevisiae* ATCC 9763. All strains were obtained from China General Microbiological Culture Collection Center and maintained on slants of Nutrient Agar (NA, Abxing, Beijing, China) for bacteria and Yeast Peptone Dextrose Agar (YPDA, Abxing, Beijing, China) for the yeast at 4 °C.

Active cultures were prepared by transferring a loop of cells from the agar slant to a test tube containing 5 ml of Nutrient Broth for bacteria and YPD Broth for the yeast. They were then incubated overnight to the logarithmic phase of growth at 37 °C for bacteria (6–10 h) and 30 °C for the yeast (12–16 h). Culture purity was examined by streaking each culture on plates of Nutrient Agar for bacteria and YPD Agar for yeast (Gilles et al., 2010). The turbidity of the cell suspensions was measured at 600 nm and adjusted to the required concentration of 10^5–10^6 CFU/ml using the McFarland standard (Firuzi, Asadollahi, Gholami, & Javidnia, 2010).

2.2.2. Agar disk diffusion assay

The EOs were screened for antimicrobial activity using the agar disk diffusion method (Rota, Carraminana, Burillo, & Herrera, 2004) against 4 microorganisms. Nutrient Agar and YPD Agar were sterilized in an autoclave and cooled to 45–50 °C before being poured into 90 mm Petri dishes. After solidifying, sterile blank filter disks (6 mm diameter) containing 5 μl of each EO were applied to the surface of agar plates that were previously seeded by spreading of 200 μl overnight fresh inoculums suspension (logarithmic growth phase cells). One standard antibiotic (kanamycin sulfate) and three chemical preservatives (sodium benzoate, potassium sorbate and methylparaben) were used as positive control. The inoculated plates were incubated for 24 h at 37 °C for bacterial and 48 h at 30 °C for yeast. Microbial inhibition was visually appraised as the diameter of the inhibition zones surrounding the disks (disk diameter included) and recorded in millimeter. The diameters of the inhibition zones were measured with a digital caliper. The agar disk diffusion tests were performed in triplicate.

2.2.3. Determination of minimal inhibitory concentration

Minimal inhibitory concentration (MIC) is cited by the most researchers as a measure of the antimicrobial performance of EOs. Bacteria and yeast sensitive to the EOs in disk diffusion assay were studied for their MIC values with some modifications of the method described by previous study (Weerakkody, Caffin, Turner, & Dykes, 2010).

The inoculums were prepared from overnight broth cultures (logarithmic growth phase cells) and suspensions were adjusted to the required microbial density (1×10^8 CFU/ml) using an Ultraspec 2000 spectrophotometer at 600 nm. After adding 20 μl of EOs to the first tube containing 4 ml of broth, serial two-fold dilutions were made in a concentration range of 0.039–5 μl/ml in 10 ml sterile test tubes containing Nutrient broth for bacteria and YPD broth for the yeast. A 400 μl suspension (1×10^8 CFU/ml) of tested microorganisms was added to each tube. A negative control tube contained broth and microorganism. Meanwhile, a positive control tube contained 50 μg/ml of kanamycin sulfate in broth and microorganism. MIC was defined as the concentration in the lowest serial dilution of the EOs which resulted in the lack of visible microorganism growth in tubes after 24 h (bacteria) and 48 h (yeast) (Reza, Rahman, Lee, & Kang, 2010).