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Original Article

Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria

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ABSTRACT

The aim of this study was to compare the antibacterial effects of several essential oils (EOs) alone and in combination against different Gram-positive and Gram-negative bacteria associated with food products. Parsley, lovage, basil, and thyme EOs, as well as their mixtures (1:1, v/v), were tested against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium*. The inhibitory effects ranged from strong (thyme EO against *E. coli*) to no inhibition (parsley EO against *P. aeruginosa*). Thyme EO exhibited strong (against *E. coli*), moderate (against *S. typhimurium* and *B. cereus*), or mild inhibitory effects (against *P. aeruginosa* and *S. aureus*), and basil EO showed mild (against *E. coli* and *B. cereus*) or no inhibitory effects (against *S. typhimurium*, *P. aeruginosa*, and *S. aureus*). Parsley and lovage EOs revealed no inhibitory effects against all tested strains. Combinations of lovage/thyme and basil/thyme EOs displayed antagonistic effects against all bacteria, parsley/thyme EOs against *B. cereus*, *S. aureus*, *P. aeruginosa*, and *E. coli*, and lovage/basil EOs against *B. cereus* and *E. coli*. Combinations of parsley/lovage and parsley/basil EOs exhibited indifferent effects against all bacteria. The combination of lovage/basil EO showed indifferent effect against *S. aureus*, *P. aeruginosa*, and *S. typhimurium*, and the combination parsley/thyme EO against *S. typhimurium*. Thyme EO has the highest percentage yield and antibacterial potential from all tested formulations; its combination with parsley, lovage, and basil EOs determines a reduction of its antibacterial activity. Hence, it is recommended to be used alone as the antibacterial agent.

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1. Introduction

Herbs have been used since ancient times for their medicinal or aromatic properties [1]. The increased interest in the use of natural preservatives as an alternative to chemical ones has brought renewed attention to the aromatic plants [2]. Lately, their bioactive compounds, essential oils (EOs), are used in active food packaging formulations for preservation purposes [3,4]. EOs can be extracted from different parts of herbs by several techniques, as water or steam distillation, solvent extraction, expression under pressure, supercritical fluid extraction, and subcritical water extraction [5]. These contain a wide variety of plant secondary metabolites that can inhibit or slow the growth microorganisms [6,7]. The main constituents of EOs are mono- and sesquiterpenes, along with carbohydrates, phenols, alcohols, ethers, aldehydes, and ketones, which are responsible for the biological activity of aromatic and medicinal plants as well as for their fragrance [8]. Oxygenated terpenoids (e.g., alcohols and phenolic terpenes) manifest the highest antimicrobial activity, but some hydrocarbons also display antimicrobial effects. Interactions between these types of compounds may lead to antagonistic, additive, or synergistic effects. The minor components are crucial to these effects [5].

Parsley, lovage, basil, and thyme are few of the aromatic herbs commonly used in Romania. These easy-to-grow plants have low costs of production. Different parts of these herbs (e.g., leaves, flowers, stems, fruits, and seeds) have been used to extract EOs. There are several studies that reveal their antibacterial activities against various bacterial strains (see supplemental online material, Tables S1 and S2). Yet, the efficiency of their mixtures against potential foodborne pathogens and spoilage bacteria has not been as yet fully studied.

In this regard, the antibacterial properties of two species of Lamiaceae (*Ocimum basilicum* and *Thymus vulgaris*) and two of Apiaceae (*Petroselinum crispum* and *Levisticum officinale*) against some Gram-positive and Gram-negative bacteria were studied in the present research. Four EOs (extracted from parsley, lovage, basil, and thyme dried leaves) were evaluated for their antibacterial activities individually and then in combination using five different *in vitro* models. To the extent of our knowledge, this is the first work to investigate the antibacterial potential of these EO mixtures. Particular attention has been paid to their synergistic, additive, indifferent, or antagonistic effects on four potential foodborne pathogens (*Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*) and one spoilage bacteria (*Pseudomonas aeruginosa*). To this intent, two antimicrobial susceptibility tests were used: the Kirby–Bauer disk diffusion test (for measuring zone diameters of bacterial growth inhibition) and the resazurin microtiter plate-based antibacterial assay [to determine the minimum inhibitory concentration (MIC)].

2. Materials and methods

2.1. Plant materials and EO extraction

Dried leaves of parsley, lovage, basil, and thyme were purchased from a Romanian company. EOs were extracted by hydrodistillation (50 g of dried leaves with 750 mL distilled water) using a Clevenger-type apparatus (for 3 hours). The extracts were dried over anhydrous sodium sulfate and stored at 4°C until analysis. The extraction yield was calculated as the volume of oil (mL) per dried leaves weight (g) and multiplied by 100. EO mixtures were prepared as follows: (1) parsley/lovage EO—parsley EO/lovage EO, 1:1 (v/v); (2) parsley/basil EO—parsley EO/basil EO, 1:1 (v/v); (3) parsley/thyme EO—parsley EO/thyme EO, 1:1 (v/v); (4) lovage/basil EO—lovage EO/basil EO, 1:1 (v/v); (5) lovage/thyme EO—lovage EO/thyme EO, 1:1 (v/v); and (6) basil/thyme EO—basil EO/thyme EO, 1:1 (v/v).

2.2. Bacterial strains

The following microorganisms were tested: *B. cereus* (ATCC 11778), *S. aureus* (ATCC 6538P), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), and *Salmonella typhimurium* (ATCC 14028). Each strain was grown in a test tube containing 45 mL sterile nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 24 hours (except *B. cereus*, which was grown at 30°C for 24 hours). The purity of the inoculum was confirmed by plating on appropriate selective media and microscopic examination of the Gram-stained smear (Optika microscope, B-252, M.A.D; Apparecchiature Scientifiche, Milan, Italy). A loopful of inoculum was transferred by streaking onto a selective medium: (1) MYP agar supplemented with Egg Yolk Emulsion and Polymyxin B (Oxoid Ltd.) for *B. cereus*; (2) Baird–Parker agar base supplemented with Egg Yolk Tellurite Emulsion (Oxoid Ltd.) for *S. aureus*; (3) Pseudomonas-agar P, base (Merck KGaA, Darmstadt, Germany) for *P. aeruginosa*; (4) TBX agar (Oxoid Ltd.) for *E. coli*; and (5) XLD agar (Oxoid Ltd.) for *S. typhimurium*. Plates were incubated for 24 hours at 30°C (*B. cereus*) or 37°C (*S. aureus*, *P. aeruginosa*, *E. coli*, and *S. typhimurium*). Bacterial morphology was confirmed by optical microscopy. Several colonies were collected with a sterile inoculating loop, transferred into sterile saline solution, and adjusted to the desired concentration using the McFarland nephelometer standards [9].

2.3. Agar diffusion susceptibility testing

EOs and their mixtures were assessed against all bacteria using the Kirby–Bauer disk diffusion test (9-mm sterile paper disks; ANTF-009-1K0; PRAT DUMAS, Couze-St-Front, France). Gentamicin was used as positive control (0.04 mg/mL in saline solution). One hundred microliters of inoculum (1.5×10^8 CFU/mL) was dispersed over the entire surface of the Mueller–Hinton agar plate (Sifin Diagnostics GmbH, Berlin, Germany) using a Drigalski spatula. A sterile paper disk was placed in the middle of a Petri dish. Then, 40 μL EO or gentamicin was released on the paper disk. Plates were incubated for 24 hours at 30°C (*B. cereus*) or 37°C (*S. aureus*, *P. aeruginosa*, *E. coli*, and *S. typhimurium*). A digital caliper was used to measure the inhibition zone diameter (in millimeters). Three replicates were run for each EO/mixture.

2.4. Broth microdilution susceptibility testing

The MIC was determined using the resazurin microtiter plate-based antibacterial assay. One part of EO was dissolved in