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Mixture design of *Origanum compactum*, *Origanum majorana* and *Thymus serpyllum* essential oils: Optimization of their antibacterial effect☆

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ABSTRACT

Several studies have revealed the efficiency of essential oils as natural antimicrobial agents in foods. However, limited reports describe the synergistic or antagonistic effects of their combinations. The aim of this study was to investigate the combined antibacterial effect of three Moroccan essential oils (*Origanum compactum*, *Origanum majorana* and *Thymus serpyllum*) and to predict the optimal combination using the mixture design approach coupled to microdilution assay, for the first time to our knowledge. The chemical composition of the oils under study was also explored.

The experimental antibacterial activity exhibited by the essential oils mixtures depended on the proportion of each oil in the combination and on the target strain. Moreover, the response surface analysis showed significant synergistic effects in some binary and ternary mixtures. The optimal mixture predicted against *Bacillus subtilis* and *Staphylococcus aureus* corresponded to 28%, 30% and 42% of *O. compactum*, *O. majorana* and *T. serpyllum*, respectively. While the optimal mixture predicted against *Escherichia coli* was composed by *O. compactum* and *O. majorana* essential oils at 75% and 25%, respectively. Our findings demonstrated the usefulness of mixture design in the estimation of antibacterial interaction of essential oils. The synergistic effect showed for some combinations may contribute to their successful application as natural preservatives in foods.

1. Introduction

Nowadays, there is an outcry worldwide on the use of synthetic preservatives in foods due to their toxicity and their adverse effects on the consumer health and/or the environment (Chivandi et al., 2016). Concomitantly, the emergence of multidrug resistance to antimicrobial agents is one of the greatest fears among public health (López-Pueyo et al., 2011). Hence, natural antimicrobial agents are attracting increasing attention. Essential oils are complex mixture of volatile compounds extracted from aromatic plants. They are endowed with several biological activities, such as antimicrobial and antioxidant properties. Thus, antibacterial essential oils have gained an increased interest and are considered as safe and eco-friendly alternative to control food-borne bacteria and other pathogenic microorganisms, notably drugs-resistant ones (Yap et al., 2014, 2013). However, it has been reported that higher concentrations of essential oils are required, in foods, to achieve similar antibacterial effects as those demonstrated with *in vitro* assays (Bassolé and Juliani, 2012; Hyldgaard et al., 2012). These high concentrations can alter the organoleptic characteristics of food and/or cause toxic (Bassolé and Juliani, 2012; Burt, 2004; Hammer and Carson, 2011). Therefore, many researchers

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have focused on studying the combined effects of essential oils in order to increase their efficiency thereby decreasing the effective dose (Gutierrez et al., 2008; Tserennadmid et al., 2010). It has been shown that the combination of terpenic compounds either in single essential oil or their mixtures affects different biochemical processes of the target bacteria, and produces various interactive antibacterial effects (Burt, 2004). For instance, synergism has been observed between the essential oils of *Origanum vulgare* and that of *Rosmarinus officinalis* against *Listeria monocytogenes* and *Yersinia enterocolitica* (de Azeredo et al., 2011). Moreover, Chinese cinnamon and cinnamon bark essential oils exhibited additive antibacterial effect against four pathogenic bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus and Salmonella Typhimurium*) (Ghabraie et al., 2015). However, limited studies have been conducted on the antimicrobial interaction between more than two essential oils.

Several methods are used to assess the antimicrobial interaction between essential oils. In checkerboard assay, the interaction is assessed by calculation of the fractional inhibitory concentration index (Bassolé and Juliani, 2012; Ouedrhiri et al., 2015; Tserennadmid et al., 2011). But this method can only assess binary combination. Besides, the time-kill test, which is often used to distinguish the bacteriostatic/bactericidal effects and the time-dependent/concentration-dependent effects, can also be used for this purpose (White et al., 1996). Nevertheless, these two methods could not always define the optimal mixture of essential oils.

The mixture design is a class of the response surface experiments in which the independent variables are the proportions of the components under investigation (Goupy and Creighton, 2006). The dependent variable (response) depends only on the proportions of the mixture components (Goupy and Creighton, 2006). The mixture design studies aim not only to develop better or innovative formulations providing optimal requests, but also to create general conceptions about responses and interactions between independent factors (Maia et al., 2011). Although the mixture design allows the modelization of the studied interaction, to our knowledge, its use to study the antibacterial effect of essential oils combinations has not been previously reported.

In this context, the present study was carried out in order to promote the development of effective preservatives using essential oils for foods and cosmetics. It aimed to evaluate the antibacterial combined effect of essential oils extracted from three Moroccan plants (*Origanum compactum* (oregano), *Origanum majorana* (sweet marjoram) and *Thymus serpyllum* (wild thyme)) and to determine the combination that provides the optimal inhibitory effect against each bacterial strain as well as the combination providing the optimal outcome against all studied strains. For this purpose, the augmented simplex-centroid mixture design (Goupy and Creighton, 2006) was used to build polynomial models describing the relationship between the antibacterial effect against each bacterial strain and the proportion of each essential oil.

2. Material and methods

2.1. Plant material and essential oils extraction

Aerial parts (leaves and stems) of three plants belonging from *Lamiaceae* family; *Origanum compactum*, and *Thymus serpyllum* were collected in August 2012, and *Origanum majorana* in February 2012 from Taounate region (Morocco). Voucher specimen of each plant was deposited at the herbarium of the National Agency of Medicinal and Aromatic Plants (NAMAP), Morocco.

To obtain essential oils the fresh aerial part (leaves and stems) of each plant were subjected separately to hydrodistillation for 3 h using a Clevenger apparatus. The obtained essential oils were stored at 4 °C in dark until use.

2.2. Gas chromatography–mass spectrometry (GC/MS) analysis conditions

The analytical GC/MS system used was an Agilent GC–MSD system (Agilent Technologies 6850/5973) with helium (high purity) as the carrier gas at a constant linear velocity of 36 cm/s. The transfer, source and quadrupole temperatures were 245 °C, 230 °C and 150 °C respectively, operating at 70 eV ionization energy and scanning the m/z range 50–550. The column used was an Agilent DB5 ms capillary column (30.0 m × 0.25 mm × 0.25 μm film thickness) programmed from 60 °C to 245 °C at 3 °C/min. Essential oil samples were diluted with hexane (Sigma Aldrich) (1:3000). The injected volume was 2.0 μL, in mode splitless, and the injector temperature was 250 °C. Identification of the individual components was based on: comparison with NIST MS Search database 2012 where possible and by Adams terpene library (Adams, 2007).

2.3. Antibacterial assays

2.3.1. Agar-disc diffusion method

The essential oils were first screened for their antibacterial activity against four bacterial strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 3366 and *Pseudomonas aeruginosa* ATCC 27853) using agar-disc diffusion method according to CLSI guidelines (CLSI, 2012) with some modifications. Briefly, fresh bacterial suspension prepared in sterile saline and adjusted to 0.5 McFarland was used to inoculate Mueller Hinton Agar plates (Biokar diagnostics, Beauvais, France). Then, sterile paper discs (6 mm in diameter) were applied on the surface of each plate and impregnated with 10 μL of essential oil. Plates were placed at 4 °C for 2 h and then incubated at 37 °C for 18–24 h. The diameters of inhibition zones were measured in millimeters. All the experiments were carried out in triplicate.

2.3.2. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations of essential oils were evaluated using the microdilution method, as described by (Haba et al., 2014) with slight modifications. In this test, serial twofold dilutions, ranging from 4 to 0,031% (v/v) of the essential oils were prepared in Mueller Hinton Broth (MHB) (Oxoid, Madrid, Spain), supplemented with bacteriological agar (Biokar diagnostics, Beauvais, France) at 0.15% (w/v). Then, the bacterial suspension (prepared in the same medium) was added to each well at a final concentration of 10^6 CFU/mL. Microplates were then incubated at 37 °C for 18 h. After the incubation period, 10 μL of resazurin (Sigma Aldrich, Germany) were added to each well to assess active bacterial growth. After further incubation at 37 °C for 2 h, the MIC value was determined as the lowest essential oil concentration that prevented a change in resazurin color. Active microbial growth was detected by reduction of blue dye resazurin to pink resorufin. Experiments were conducted in triplicate and modal values were considered.

2.3.3. Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentrations were determined by spreading 5 μL from negative wells on Luria Bertani agar plates (10 g/L of tryptone (Difco), 5 g/L of yeast extract (Difco), 10 g/L of NaCl (Sigma Aldrich) and 20 g/L of bacteriological agar (Biokar)). The MBC value corresponded to the lowest concentration of the essential oil yielding negative subculture after incubation at 37 °C for 24 h.