



Combination of sweet orange, lentisk and lemon eucalyptus essential oils: Optimization of a new complete antimicrobial formulation using a mixture design methodology

Samiah Hamad Al-Mijalli^a, Mohamed Jeddi^b, Naoufal El Hachlafi^b,
Emad M. Abdallah^c, Hamza Assaggaf^d, Ahmed Qasem^d, Bodour S. Rajab^d,
Learn-Han Lee^{e,f,*}, Abdelhakim Bouyahya^{g,**}, Khang Wen Goh^h,
Long Chiau Ming^{i,***}, Hanae Naceiri Mrabti^{j,k}

^a Department of Biology, College of Sciences, Princess Nourah Bint Abdulrahman University, Riyadh 11671, Saudi Arabia

^b Laboratory of Microbial Biotechnology and Bioactive Molecules, Faculty of Sciences and Technologies Faculty, Sidi Mohamed Ben Abdellah University, P.O. Box 2202, Imouzzar Road, Fez, Morocco

^c Department of Science Laboratories, College of Science and Arts, Qassim University, Ar Rass 51921, Saudi Arabia

^d Department of Laboratory Medicine, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah 21955, Saudi Arabia

^e Sunway Microbiomics Centre, School of Medical and Life Sciences, Sunway University, Sunway City 47500, Malaysia

^f Novel Bacteria and Drug Discovery Research Group (NBDD), Microbiome and Bioresource Research Strength (MBRS), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Sunway City, Malaysia

^g Laboratory of Human Pathologies Biology, Department of Biology, Faculty of Sciences, Mohammed V University in Rabat, 10106, Morocco

^h Faculty of Data Science and Information Technology, INTI International University, 71800 Nilai, Malaysia

ⁱ School of Medical and Life Sciences, Sunway University, Sunway City 47500, Malaysia

^j Laboratory of Pharmacology and Toxicology, Bio Pharmaceutical and Toxicological Analysis Research Team, Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco

^k High Institute of Nursing Professions and Health Techniques of Casablanca, Casablanca, Morocco

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ABSTRACT

Sweet orange (*Citrus × sinensis* (L.) Osbeck), lentisk (*Pistacia lentiscus* L.) and lemon eucalyptus (*Eucalyptus citriodora* Hook) are medicinal plants known by its culinary virtues. Their volatile oils have demonstrated promising antimicrobial activity against a panel of microbial strains, including those implicated in food deterioration. In this exploratory investigation, we aimed to determine the antimicrobial formulation of sweet orange, lentisk and lemon eucalyptus essential oils (EOs) using the simplex-centroid mixture design approach coupled with a broth micro-dilution method. EOs were first extracted by hydrodistillation, and then their phytochemical profile was characterized using Gas chromatography–mass spectrometry (GC-MS). GC-MS analysis identified D-limonene (14.27%), careen-3 (14.11%), β-myrcene (12.53%) as main components of lentisk EOs, while lemon eucalyptus was dominated by citronellal (39.40%), β-citronellol

* Corresponding author. Sunway Microbiomics Centre, School of Medical and Life Sciences, Sunway University, Sunway City 47500, Malaysia.

** Corresponding author.

*** Corresponding author.

E-mail addresses: shamejale@pnu.edu.sa (S.H. Al-Mijalli), mohamed.jeddi@usmba.ac.ma (M. Jeddi), naoufal.elhachlafi@usmba.ac.ma (N. El Hachlafi), emad100sdl@yahoo.com (E. M. Abdallah), hmsaggaf@uqu.edu.sa (H. Assaggaf), aaqasem@uqu.edu.sa (A. Qasem), bsrajab@uqu.edu.sa (B. S. Rajab), learnhanl@sunway.edu.my (L.-H. Lee), a.bouyahya@um5r.ac.ma (A. Bouyahya), chiaumingl@sunway.edu.my (L.C. Ming), naceiri.mrabti.hanae@gmail.com (H.N. Mrabti).

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(16.39%) and 1,8-cineole (9.22%). For sweet orange EOs, D-limonene (87.22%) was the principal compound. The three EOs exhibited promising antimicrobial potential against various microorganisms. Lemon eucalyptus and sweet orange EO showed high activity against most tested microorganisms, while lentisk EO exerted important effect against some microbes but only moderate activity against others. The optimization formulations of antimicrobial potential showed interesting synergistic effects between three EOs. The best combinations predicted on *C. albicans*, *S. aureus*, *E. coli*, *S. enterica* and *B. cereus* correspond to 44%/55%/0%, 54%/16%/28%, 43%/22%/33%, 45%/17%/36% and 36%/30%/32% of *Citrus sinensis*, *Pistacia lentiscus* and *Eucalyptus citriodora* EOs, respectively. These findings suggest that the combination of EOs could be used as natural food preservatives and antimicrobial agents. However, further studies are needed to determine the mechanisms of action and efficacy of these EOs against different microorganisms.

1. Introduction

The use of medicinal plants dates back to ancient times, where plants were considered the primary source of medicine to treat different illnesses. Historically, medicinal plants have been utilized in different cultures, including the Greek, Roman, Chinese, and Indian [1–3]. The Greek physician Hippocrates, also known as the father of medicine, listed the use of over 400 plants for medicinal purposes [4]. Similarly, the Indian Ayurvedic system of medicine has been using plants for over 5000 years [5]. Plants such as *Aloe vera* (L.) Burm. F., *Cannabis sativa* L., and *Papaver somniferum* L. have been used for their medicinal properties for centuries [1]. With the advance of conventional medicine, the use of medicinal plants has declined. However, recent reports have shown that medicinal plants can still provide a source of novel drugs to counteract the evolution of several chronic ailments, including cancer, diabetes, and infections [6]. The rise of antimicrobial resistance (AMR) has become a major public health concern worldwide, with a growing need to find novel and effective antimicrobial agents [7]. Current antibiotics are becoming less effective, and few new drugs are being developed to replace them. According to the World Health Organization, AMR is responsible for 700,000 deaths each year, and it is projected to become a leading cause of death worldwide by 2050 [8]. This problem is exacerbated by the overuse and misuse of antibiotics in both human and animal populations, which has led to the selection of resistant strains of bacteria. The need for new antimicrobial drugs is therefore urgent, with an emphasis on developing novel compounds that are effective against resistant bacteria [9]. Researchers are exploring new approaches, such as the use of phage therapy, bacteriocins, and antimicrobial peptides, to combat AMR. Additionally, efforts are being made to revive previously abandoned antibiotic candidates through drug repurposing and to develop new antibiotics through the exploration of natural products and synthetic chemistry. It is clear that the development of effective antimicrobial drugs is crucial in the fight against AMR and ensuring continued effective treatment of infectious diseases [10, 11].

Essential oils have been identified as a promising source of antimicrobial agents thanks to their wide range of chemical constituents and the potential for synergistic effects between these compounds [12]. Numerous volatile oils are obtained from various plant parts and possess a variety of biological activities, including antibacterial and antifungal activities making them a potential alternative to conventional antibiotics [13–15]. The wide spectrum antimicrobial potential of EOs against foodborne pathogens, encouraging their uses in the food industry, extending the storage stability of food products without any modifications of the organoleptic properties of food [16–18]. Generally, EOs are considered as a mixture of various bioactive components belonging to different chemical classes, such as monoterpenes (Hydrocarbons, oxygenated), sesquiterpene (Hydrocarbons and oxygenated). These components have demonstrated useful effects in several food-based systems, prolonging the shelf life of foods [19,20]. Moreover, vapors of EOs have been evidenced as promising constituents of active packaging. In fact, the direct application of volatile oils in the polymer films of packaging has shown to eradicate microbial deterioration and preserve the organoleptic properties of food [21,22].

The word "mixture" may indicate a variety of things depending on the situation, including how it is used and how it is produced. Additionally, we can identify binary mixes (composed of two components), ternary mixtures, and so on depending on the complexity [23,24]. In fact, the combination of these components might produce additive, synergistic or antagonistic interaction. These interactions are additive when their combinatorial action is the sum of each component independently, synergistic when the combinatorial action is greater than the sum of each component independently, while antagonistic when the combinatorial action is less than the sum of each independently [25,26].

In the last two decades, the application of mixtures in food industry has gained increasing interest. Indeed, the formulation of a giving product should consider the used components, especially their relative proportion in the mixture [27]. Using a mixture optimizing methodology could have different technological applications in food, which may increase the shelf-life of food, enhance the active packaging and the sensory properties of a baked products [27].

Sweet orange (*Citrus sinensis* (L.) Osbeck), lentisk (*Pistacia lentiscus* L.) and lemon eucalyptus (*Eucalyptus citriodora* Hook) are medicinal plants known by its culinary virtues in Morocco [28]. *C. sinensis* EOs have shown the presence of a plethora of bioactive molecules belonging to several classes, including monoterpene, sesquiterpene, ketones, alcohols, aldehydes. Limonene and myrcene were the principal identified compounds in this oil [29]. Moreover, the chemical analysis of *P. lentiscus* EOs have identified up to 245 components, with the dominance of α -pinene, limonene, terpinen-4-ol, germacrene D, α -phellandrene, and *p*-cymene [30,31]. As regards *E. citriodora*, a large number of volatile constituents were characterized in their EOs. The main compounds encountered were citronellal, citronellol, geranial and myrcene [32]. The *C. sinensis* EOs serve in the synthesis of various products, such as perfumes and certain hygiene products [33]. On the other hand, *P. lentiscus* and *E. citriodora* EOs have different application in perfumery, food and

pharmaceutical industries [34,35].

These three EOs are known by their valuable healthy benefits and pharmacological properties, including antioxidant, antiviral, antidiabetic, analgesic, and insecticidal properties. Besides, their volatile oils have demonstrated potent antimicrobial activity against a panel of microbial strains, including those implicated in food deterioration [36–38], providing scientific basis for their potential application as food preservatives. However, little is known about the synergistic antimicrobial activity of these oils using mixture design methodology. Therefore, in this exploratory study, we aimed to determine the combined antimicrobial effect of sweet orange, lentisk and Lemon Eucalyptus EOs, and to develop a general model about interactions between their components using simplex–centroid mixture design approach. To our knowledge, this is the first investigation to optimize the antimicrobial profile of sweet orange, lentisk and lemon eucalyptus EOs combination adopting simplex–centroid mixture design methodology.

2. Materials and methods

2.1. Plant material and EO extraction

Eucalyptus citriodora Hook (Leaves (12–14 cm)), *Citrus sinensis* (L.) Osbeck (Fruits (10 cm)) and *Pistacia lentiscus* L. (Leaves (8 cm)) were harvested in Mars 2022, from Taounate region (34° 32′ 09″ N, 4° 38′ 24″ W, Morocco). The botanical identification of these species was performed based to the protocol of González-Tejero et al. [39] and affirmed by ethnobotanists from Department of Botany at Scientific Institute of Rabat (University of Mohammed V), Morocco, under voucher identifiers RAB 1713–1715. The samples were dried under constant conditions in mini laminar flow hood at 38 °C using continuous Ventilation for 7 days for *P. lentiscus* and 12 days for *E. citriodora* and *C. sinensis*. These drying periods have been confirmed to be suitable in preliminary experiments to achieve the desired final moisture content of $9\% \pm 3.2\%$. EOs were obtained as follow: an amount of 100 g of dried plants (Leaves, and fruits (*C. sinensis*)) were exposed to hydro-distillation for 3–4 h using Clevenger type tool (Extraction was conducted in triplicate). Then, the EOs was desiccated with anhydrous Na₂SO₄, and thereby kept in appropriate conditions (4 °C) pending upcoming tests.

2.2. GC-MS analysis of volatile components

The volatile constituents were determined using gas chromatography (Trace GC-Ultra, S/N 20062969) (GC) coupled with HP 5975C mass spectrometer with electron control ionization (70 eV). A non-polar HP-5 MS capillary column (30 m, 0.32 mm × 0.25 µm coated with 95% dimethyl polysiloxane) was used. Purified helium (He) was serve as gas carrier (flow rate of 48 mL/s). The machine was automated with splitless injection mode. The temperature of injector and detector was 250 and 300 °C, respectively. The oven temperature was established to rise from 50 to 280 °C at a ratio of 4 °C/min. The chemical characterization of each compound was processed on the basis of their retention indices (R_{index}) relative to (C₈–C₂₄) n-alkanes series with those recorded in data libraries. In addition, identification was accomplished by matching their reported mass spectra (MS) with those described in the NIST/Wiley MS data and other available literature data [40,41]. The quantification of oil components via internal normalization of peak area without using any adjustment elements.

2.3. Microbial strains and culture preparation

In order to report the antimicrobial efficacy of EOs, we utilized five different microbial strains, including two Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* foodborne isolate), two Gram-negative bacteria (*Salmonella enterica* serotype typhi foodborne isolate and *Escherichia coli* ATCC 25922), and one yeast (*Candida albicans* clinical isolate) obtained from the Laboratory of Microbial Biotechnology and Bioactive Molecules at the Faculty of Sciences, Fez (Morocco). These microorganisms were chosen not only because it is responsible for nosocomial infection, but also by their implication in food deterioration and several infectious diseases. Prior to use, the strains were incubated on an inclined Luria-Bertani (LB) agar medium at 4 °C. bacteria were sub-cultured in LB at 37 °C for 20–24 h, while *C. albicans* was sub-cultured on Yeast Extract-Peptone-Dextrose (YPD) agar plates at 25 °C for 48 h. The antibacterial screening was performed at a final inoculum concentration of 10⁶ CFU/mL in accordance with the guidelines of the NCCLS United States [42,43].

2.4. Disc-diffusion method

The test aimed to assess the antimicrobial property of the examined EOs using the agar disc-diffusion technique with slight modifications to a previously published protocol [44]. The experiment utilized LB agar medium for bacteria and YPD agar for *C. albicans*. Microbial strains adjusted to 0.5 McFarland standard were streaked on agar plates. Each sterile 6 mm paper disc was soaked with 6 µL of pure EO and placed on the inoculated agar plates. Kanamycin (15 µg/disc) and Ketoconazole (10 µg/disc) were utilized as positive controls for bacteria and *C. albicans*, respectively. Meanwhile, DMSO at a concentration of 5% was used as negative control. Incubation was performed at appropriate conditions (37 °C for bacteria and at 25 °C for yeast). The resulting inhibitory zones were reported in millimeters, and the data were presented as the mean ± SD from three independent experiments.

2.5. Detection of MIC

This protocol aimed to report the minimum inhibitory concentration (MIC) of the examined EOs using a previously reported

method with minor modifications [13]. In brief, EO concentrations ranging from 8.0% to 0.007% (v/v), from 64 to 0.25 µg/mL (w/v) for Kanamycin and from 128 to 0.25 µg/mL (w/v) for Ketoconazole were prepared in Mueller-Hinton broth with 5% DMSO (two-fold dilution) in sterile 96-well plates. The concentration of DMSO was chosen based on previous findings that concentrations up to 7.8% had no significant impact on viable bacterial cell count [45]. Next, 10 µL of bacterial or yeast culture was added to each well, and the plates were incubated overnight at 37 °C or for 48 h at 25 °C, respectively. Mueller-Hinton broth with 5% DMSO without microbial suspension was employed as a negative control and Kanamycin and Ketoconazole were utilized as positive controls. After incubation, *p*-iodonitrotetrazoliumchloride (INT) 95% was added to all microtubes to determine bacterial growth, and the MIC was determined as the highest EOs dilution where no color change was observed. The experiments were carried out in triplicates ($n = 3 \pm \text{SD}$).

2.6. Detection of MBC and MFC

The minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) refers to the lowest concentration of giving antimicrobials that can eliminate a specific microbial strain [13]. Once the MIC assessment was completed, the MBC or MFC test was carried out. Briefly, 25 µL of each MIC tube was pipetted onto nutrient agar plates for bacteria or Sabouraud dextrose agar for yeast. The plates were then incubated overnight at 37 °C. Furthermore, the MBC/MIC values for bacteria and MBC/MFC values for *C. albicans* were indicated to categorize the EOs as bactericidal/fungicidal (≤ 4) or bacteriostatic/fungistatic (> 4). The experiments were carried out in triplicates ($n = 3 \pm \text{SD}$).

2.7. Mixture design and mathematical model

A simplex centroid design was employed to assess and optimize the ternary antimicrobial properties of the selected EOs [46]. The Factors signify the proportions of each EO in the mixture and their values can range from 0 to 1 without constraints on the design space.

The design comprised of twelve experiments and was illustrated as equilateral triangle form (Fig. 1) comprising, the vertices of the triangle (X_1 - X_2 - X_3) represent three pure products, the midpoints of the three sides are made up of the binary combinations (X_4 - X_5 - X_6) and the central point (Centroid) (X_7). This test has been performed at three replicate, and three augmented points (X_8 - X_9 - X_{10}) are related to the ternary combinations. Therefore, twelve was the total number of experiments in this design.

The responses measured in this study were the antimicrobial effects against each microbial strain. These data were then fitted to a special cubic model using the least-squares regression to determine the unknown coefficients in Eq (1).

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + \epsilon \quad (1)$$

With.

Y represents the MIC response expressed in % (v/v); β_1 , β_2 and β_3 are the coefficients of the linear terms; β_{12} , β_{13} and β_{23} are the coefficients of the binary terms; β_{123} is the coefficients of the ternary term, while ϵ is an error term.

2.8. Statistical analysis

The F-test for ANOVA was utilized to verify the significance of the fitted models. Thus, the mean square regression (MSR) and mean square residuals (MSr) were used to calculate the $F_{\text{ratio}} \text{ (G/L)}$ [47]. Besides, the calculation of the $F_{\text{ratio LOF/PE}}$ was carried out with: (MS_{LOF}) as the mean square lack of fit and (MS_{PE}) as mean square pure error, the $F_{\text{ratio LOF/PE}}$ was utilized to assess how well the model fit to the observations. High $F_{\text{LOF/PE}}$ values indicate poor model fit [48]. Furthermore, the coefficient of determination R^2 and R^2_{Adj}

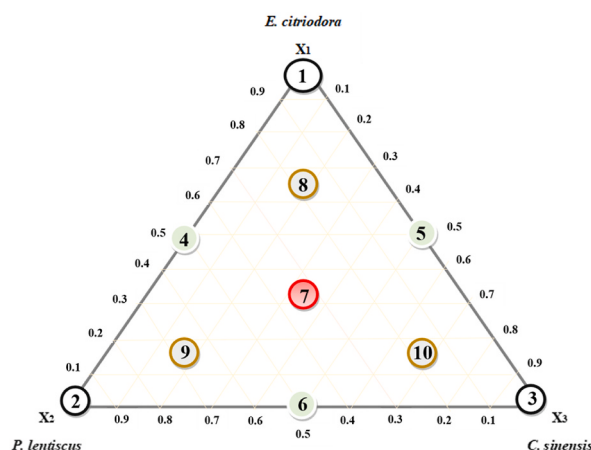


Fig. 1. An overview of the simplex centroid design for a three-component mixture. The factors X_1 , X_2 , and X_3 represent components *E. citriodora*, *P. lentiscus* and *C. sinensis*, respectively.

were used to assess the accuracy of the postulated models [49]. Student's *t*-test was utilized to assess the significance of the estimated coefficients. The experimental design conception, as well as the statistical and graphical analysis, were carried out using of Expert Design software version 11 and SAS JMP software version 14.

2.9. Optimization tools

Optimization tools were utilized as a useful method to predict the optimal combination of the three studied EOs. In this context,

Table 1

Chemical composition of *P. lentiscus*, *E. citriodora* and *C. sinensis* EOs.

No. ^a	Compounds ^b	Molecular formula	RI ^c	% Relative peak area			Identification
				<i>P. lentiscus</i>	<i>E. citriodora</i>	<i>C. sinensis</i>	
1	α -Thujene	C ₁₀ H ₁₆	924	1.42	–	1.45	MS, R _{index}
2	α -Pinene	C ₁₀ H ₁₆	939	–	1.01	1.59	MS, R _{index}
3	Camphene	C ₁₀ H ₁₆	943	–	–	–	MS, R _{index}
4	Careen-3	C ₁₀ H ₁₆	948	14.11	0.36	–	MS, R _{index}
5	α -Myrcene	C ₁₀ H ₁₆	958	–	–	4.84	MS, R _{index}
6	β -Thujene	C ₁₀ H ₁₆	968	5.57	–	–	MS, R _{index}
7	β -Pinene	C ₁₀ H ₁₆	980	0.26	1.51	–	MS, R _{index}
8	β -Myrcene	C ₁₀ H ₁₆	987	12.53	–	–	MS, R _{index}
9	α -Terpinene	C ₁₀ H ₁₆	998	3.83	–	–	MS, R _{index}
10	α -Phellandrene	C ₁₀ H ₁₆	1002	5.73	–	–	MS, R _{index}
11	ν -Limonene	C ₁₀ H ₁₆	1024	14.27	0.42	87.22	MS, R _{index}
12	1,8-cineole	C ₁₀ H ₁₈ O	1030	–	9.22	–	MS, R _{index}
13	<i>p</i> -Menth-8-ene	C ₁₀ H ₁₆ O	1031	–	–	0.40	MS, R _{index}
14	<i>o</i> -Cymene	C ₁₀ H ₁₄	1042	2.52	–	–	MS, R _{index}
15	<i>p</i> -Mentha-1,4(8)-diene	C ₁₀ H ₁₆	1052	2.74	–	–	MS, R _{index}
16	γ -Terpinene	C ₁₀ H ₁₆	1059	4.72	0.36	–	MS, R _{index}
17	2-Nonanone	C ₉ H ₁₈ O	1090	1.09	–	–	MS, R _{index}
18	2-Nonanol	C ₉ H ₂₀ O	1098	0.49	–	–	MS, R _{index}
19	Linalool	C ₁₀ H ₁₈ O	1101	–	–	1.00	MS, R _{index}
20	Limonene oxide	C ₁₀ H ₁₆ O	1134	–	–	0.49	MS, R _{index}
21	2-Norbornanol	C ₁₀ H ₁₈ O	1138	–	0.51	–	MS, R _{index}
22	<i>p</i> -Mentha-cis-2,8-dien-1-ol	C ₁₀ H ₁₆ O	1140	–	–	0.26	MS, R _{index}
23	Terpinen-4-ol	C ₁₀ H ₁₈ O	1143	7.94	–	–	MS, R _{index}
24	Citronellal	C ₁₀ H ₁₈ O	1151	–	39.40	–	MS, R _{index}
25	α -Citronellol	C ₁₀ H ₂₀ O	1161	–	4.18	–	MS, R _{index}
26	β -Citronellol	C ₁₀ H ₂₀ O	1179	–	16.39	–	MS, R _{index}
27	ν -Carvone	C ₁₀ H ₁₄ O	1190	–	–	0.44	MS, R _{index}
28	Neo-isopulegol	C ₁₀ H ₁₈ O	1196	–	8.46	–	MS, R _{index}
29	Decanal	C ₁₀ H ₂₀ O	1204	–	–	0.76	MS, R _{index}
30	Copaene	C ₁₅ H ₂₄	1221	0.39	–	–	MS, R _{index}
31	2-Undecanone	C ₁₁ H ₂₂ O	1251	1.16	–	–	MS, R _{index}
32	Fenchyl acetate	C ₁₂ H ₂₀ O ₂	1277	1.11	–	–	MS, R _{index}
33	Citronellic acid	C ₁₀ H ₁₈ O ₂	1293	–	1.71	–	MS, R _{index}
34	Neoisopulegol hydrate	C ₁₀ H ₂₀ O ₂	1320	–	2.87	–	MS, R _{index}
35	Citriodiol	C ₁₀ H ₂₀ O ₂	1321	0.53	1.08	–	MS, R _{index}
36	β -Elemene	C ₁₅ H ₂₄	1398	3.50	–	–	MS, R _{index}
37	(E)-Caryophyllene	C ₁₅ H ₂₄	1420	0.37	–	–	MS, R _{index}
38	γ -Cadinene	C ₁₅ H ₂₄	1435	3.13	–	–	MS, R _{index}
39	α -Humulene	C ₁₅ H ₂₄	1465	0.41	–	–	MS, R _{index}
40	Epizonaren	C ₁₅ H ₂₄	1469	0.53	–	–	MS, R _{index}
41	γ -Murolene	C ₁₅ H ₂₄	1474	0.78	–	–	MS, R _{index}
42	Germacrene D	C ₁₅ H ₂₄	1515	2.95	–	–	MS, R _{index}
43	δ -Cadinene	C ₁₅ H ₂₄	1534	2.66	–	–	MS, R _{index}
44	α -Cadinol	C ₁₅ H ₂₆ O	1580	0.49	–	–	MS, R _{index}
45	tau-Murolol	C ₁₅ H ₂₆ O	1581	0.67	–	–	MS, R _{index}
46	<i>m</i> -Camphorene	C ₂₀ H ₃₂	1982	0.70	–	–	MS, R _{index}
	<i>p</i> -Camphorene	C ₂₀ H ₃₂	1982	0.26	–	–	MS, R _{index}
	Total identified			99.82	93.36	98.81	
	Monoterpene hydrocarbons			67.38	2.88	95.51	
	Oxygenated monoterpenes			11.79	82.42	2.19	
	Sesquiterpene hydrocarbons			16.34	3.52	–	
	Oxygenated sesquiterpenes			1.16	–	–	
	Others (ketones, aldehydes, acids and esters)			3.15	4.92	1.16	
	Yield (% v/w)			2.46	3.04	5.22	

^a In order of elution on HP-5ms.

^b Compounds revealed based on RI and MS.

^c Retention index calculated from alkanes series on HP-5 MS capillary column (C₈–C₂₄).ND: Not determined (–).

augmented Simplex-centroid design was employed to assess the ternary antimicrobial effect of the studied EOs. The optimal formulation of essential oils was identified by contour and surface plots adopted from iso-response curves, resulting in a compromise of responses [50]. Then, the “desirability” function was performed to precisely identify the desired response according to the optimal conditions. This function allows to precise optimal adjustment with a rate ranging from 0% to 100%. A score of 100% is assigned when the system produces the best possible desired response, while a value of 0% represents an undesirable response [50,51].

3. Results and discussion

3.1. Identification of volatile constituents

The essential oil yields (% v/w) for *P. lentiscus*, *C. sinensis*, *E. citriodora* EOs were 2.46%, 5.22%, and 3.04%, respectively. These EOs were pale yellow in color for *C. sinensis*, light-brown for *P. lentiscus*, and yellow for *E. citriodora*.

The chemical characterization of *P. lentiscus*, *C. sinensis*, *E. citriodora* EOs, including the percentage of each constituent, molecular formula, total peak area and retention index are presented in Table 1. As shown, a total of twenty-nine components were revealed in *P. lentiscus* EO, which accounted for approximately 99.82% of total identified compounds. *P. lentiscus* EO was mainly represented by monoterpene hydrocarbons (67.38%). The minor oil fractions were characterized by sesquiterpene hydrocarbons (16.34%), oxygenated monoterpenes (11.79%) and oxygenated sesquiterpenes (1.16%). The major compounds identified in this EO were α -limonene (14.27%), careen-3 (14.11%), β -myrcene (12.53%) and terpinen-4-ol (7.94%).

The phytochemical profile of *P. lentiscus* EO has been widely described in the literature. Indeed, different EO chemotypes have been identified. The main components encountered were: α -pinene (25%), terpinen-4-ol (21%) and β -caryophyllene (19%) in lentisk EO originated from southern Italy (Apulia) [52]; germacrene D (22%), α -pinene (17%), and thujene (15%) in Moroccan lentisk EO [53]; α -pinene (17%) and 4-terpineol (11.93%) in Tunisian *P. lentiscus* EO [36]; and tricyclene (7%), 4-terpineol (7%) and thujene (6.01%), in EO of *P. lentiscus* harvested from Algeria [54]. As evidence in the literature, there are significant quantitative and/or qualitative fluctuations in the chemical constituents of *P. lentiscus* EO depending on phenological stages of plant. In fact, at vegetative stage (February in Turkey), *P. lentiscus* EO has been designated as β -myrcene (39%) [34] and terpinen-4-ol (29%) chemotypes [55]. At flowering stage, several chemotypes has been described, including myrcene (33.46%) and α -pinene (19.20%) [56], germacrene D (22%) and α -pinene (17%) [53], as well as terpinen-4-ol (12%) chemotype [36]. At fruiting stage, Ismail and his colleagues [22] detected α -pinene (20%), limonene (15%), β -pinene (9%) as main compounds of lentisk growing in Tunisia. Another investigation showed that EO obtained by hydrodistillation from the leaves of an Algerians lentisk has characterized by its richness of limonene (43%), α -pinene (34%), and myrcene (33.1%) [16]. However, Hamiani et al. [57] identified terpinen-4-ol (41%) as main components of *P. lentiscus* EO collected from west Algeria.

Interestingly, Yosr et al. [58] investigated the variation in the volatile components of lentisk EOs (Leaves) depending on the plant sex for different phenological stages (four harvesting times) in Tunisia (Ezzit Djebel Mountain region). Female plant mainly composed of limonene (26–29%) at the early fruiting (June–August) and late fruiting (October) periods. While, at the at the flowering and early fruiting stages, germacrene D (20%) was identified as main compounds in lentisk EO. Moreover, the highest amount of δ -cadinene (15.6%) has been noted at the flowering (March) period [58]. For male trees, lentisk EO has been characterized by its abundance of germacrene D (13%) at the flowering stage. As indicated by above mentioned data, ecological factors support substantial part of the interpretation regarding difference between our findings and literature data.

As regards, *E. citriodora* EO, a total of 14 components, accounting for 93.36% of the volatile oil, were detected. These compounds are specifically monoterpenoid in the nature. Oxygenated monoterpenes (82.42%) represented the major portion of this oil, while sesquiterpene hydrocarbons (3.52%) and monoterpene hydrocarbons (2.88%) represented were little represented in *E. citriodora* EO. The main constituents identified were citronellal (39.40%), β -citronellol (16.39%) and 1,8-cineole (9.22%). Despite there are various investigations highlighted the health benefits and pharmacological effects of *E. citriodora*, the data on the chemical constituents of their volatile oils need to be further explored. Generally, *E. citriodora* EO has known by its richness of citronellal (83.50%) [32]. In another study, the chromatographic analysis using GC-MS tool of *E. citriodora* EO collected from Northern Thailand identified 50 different constituents, representing 99% of the total oil content [38]. The major detected constituents were citronellal (60.55%) followed by dl-isopulegol (10.57%) and citronellol (9%) [38].

For *C. sinensis* EO, 10 volatile compounds have been identified, which represented a portion of 98.81%. This oil is highly predominated by monoterpene hydrocarbons (95.51%), whereas a minor amount of oxygenated monoterpenes (2.19%) has been revealed. α -limonene was the principal chemical characterized in *C. sinensis* EO, with a high percentage 87.22%. Moreover, other components have also identified small proportions, including α -myrcene (4.84%), α -pinene (1.59%), α -thujene (1.45%) and linalool (1.00%). As indicated in the literature, the phytochemical content of *C. sinensis* EO have gained much focus. Matuka et al. [59] investigated the chemical variations of South African *C. sinensis* EO from leaves and peels (fresh and dried) using GC-MS tool. The results showed that thujene (20.4%) and 4-terpineol (13.2%) are the main components of fresh leaves EO, whereas, β -elemene (16.3%) and thujene (10.7%) were detected in dried leaves. Concerning, fresh and dried peels EO, 24 and 25 components, representing 99.3% and 99.4% respectively, were revealed, with limonene (80.5–73.6%) was the main predominant constituent [59]. These findings showed that air-drying of the leaves materiel of *C. sinensis* may impact the volatile oil constituent pattern.

On the other hand, study carried out on Nigerian *C. sinensis* dried peels EO identified the appearance of other compounds, including spathulenol (9.97%) and cymene (2.09%) which were not detected in our study [60]. Moreover, other compounds have been characterized in EO of *C. sinensis* grown in Egypt, which elucidating 4-terpineol (13.2%) and limonene (7.5%) as major compounds [61].

The chemical polymorphism of these species may explained by several intrinsic and extrinsic factors, including local environmental

conditions (climate, soil composition (Zn, Fe, Cu)), seasonal variations, phenological stages, plants part used could justify. Moreover, the genetic background of the varieties of these medicinal plants could also affect the qualitative and quantitative constituents of the volatile oils. These factors could affect the synthesis and release of volatile components, by targeting specific enzymes.

3.2. Single antimicrobial action

In the current investigation we aimed to assess the antimicrobial potential of three essential oils using various tests including the disc diffusion technique. The tested EOs were *E. citriodora*, *C. sinensis*, and *P. lentiscus*. The inhibition zone diameter, which measures the antimicrobial activity of the EOs against susceptible microorganisms, was classified into three categories: weak activity (10 mm or less), moderate activity (10–15 mm), and high activity (15 mm or more) [62]. Accordingly, as shown in (Table 2), findings discovered that all EOs test exhibited remarkable antimicrobial activity with varying degrees. *E. citriodora* EO demonstrated high activity on all tested microorganisms, with the highest inhibition zone diameter recorded for *Bacillus cereus* (22.34 ± 0.6 mm). The EOs also showed high action against *Candida albicans* (20.85 ± 0.67 mm), *Salmonella enterica* (18.06 ± 1.18 mm), *Staphylococcus aureus* (16.13 ± 1.5 mm), and *Escherichia coli* (15.25 ± 0.03 mm), respectively. *C. sinensis* EO also exhibited high activity against *B. cereus* (26.16 ± 4.04 mm), *S. aureus* (17.45 ± 1.12 mm), and *C. albicans* (18.05 ± 2.01 mm), respectively. However, the EO showed only moderate activity against *S. enterica* (14.05 ± 0.28 mm) and *E. coli* (12.01 ± 0.5 mm), respectively. *P. lentiscus* EO recorded high activity against *S. aureus* (21.23 ± 2.5 mm), *E. coli* (16.07 ± 1.53 mm), *B. cereus* (15.18 ± 1.15 mm), and *C. albicans* (15.5 ± 0.75 mm), respectively. However, the EO exhibited only moderate activity against *S. enterica* (10.11 ± 0.72 mm). To determine if there were any statistical differences in the antimicrobial activity of the three essential oils, an ANOVA (analysis of variance) test was conducted. ANOVA analysis indicated significant differences ($p < 0.05$) in the antimicrobial activity of the three EOs against all tested microbial strains.

The effectiveness of EOs in inhibiting or killing microorganisms was evaluated using MIC, MBC, and MFC tests. These tests determine the minimum concentration of an antimicrobial agent required to inhibit or kill a particular microorganism. The lower the values obtained from these tests, the more effective the antimicrobial agent is against the microorganism. The results presented in Table 3 indicate that *E. citriodora* EO was the most effective of the three EOs tested, showing the lowest MIC, MBC, and MFC values.

For *C. albicans*, the least MIC and MBC values recorded was 0.125% v/v, while the least MBC and MFC values recorded were 0.25% for *S. aureus* and *S. enterica*, and 0.5% for *E. coli* and 0.5% and 1.0% for *B. cereus*, respectively. *P. lentiscus* EO was found to be less effective than *E. citriodora* EO. The least MIC, MBC, and MFC values recorded were with *C. albicans* (0.5%), followed by *S. aureus* (1.0%), *B. cereus* (1.0% and 4.0%), and *S. enterica* (2.0%). The highest MIC and MBC values were recorded with *E. coli* (4.0% and 8.0%, respectively). *C. sinensis* EO was also effective against the tested microorganisms. The least MIC, MBC, and MFC values recorded were with *C. albicans* (0.125%), followed by *B. cereus* (0.5%), *S. aureus* (0.5% and 2.0%), *S. enterica* (1.0%), and *E. coli* (2.0%). The MBC/MIC and MFC/MIC ratios were equal to or less than 4.0 for all three EOs, indicating a possible bactericidal and fungicidal mechanism. This means that the EOs were effective in killing the microorganisms rather than simply inhibiting their growth.

The disc-diffusion test is a preliminary screening method used to determine the antimicrobial activity of a compound or agent against a range of microorganisms but it only provides a measure of the susceptibility of the microorganism to the antimicrobial agent. It does not provide full antimicrobial information [63]. Therefore, MIC, MBC and MFC tests are necessary to show the minimum inhibitory, bactericidal or fungicidal concentration required to completely inhibit or kill the tested microorganisms. The MIC test provides more quantitative data about the effectiveness of an antimicrobial agent and can help determine the appropriate dosage and treatment duration needed to effectively treat an infection. The MBC test, on the other hand, provides information about the efficacy of the antimicrobial agent in completely eliminating the microorganism, which is crucial for preventing the development of antibiotic resistance and recurrent infections [64,65]. All the tested EOs exhibited significant antimicrobial activity, but the degree of activity varied among the tested microorganisms. *E. citriodora* oil showed the highest activity against all tested microorganisms, followed by *C. sinensis* and *P. lentiscus* oils. Several previous studies have reported the antimicrobial activity of *E. citriodora* oil. For instance, According to prior research, *E. citriodora* essential oil has significant antibacterial action against Gram-positive bacteria compared to Gram-negative bacteria, as well as efficacy against drug-resistant *C. albicans* and *E. coli* mutants [66], which is in agreement with the

Table 2
Antimicrobial activity of *E. citriodora*, *P. lentiscus*, *C. sinensis* EOs using disc diffusion method.

Bacteria	Gram-strains	Diameter of Inhibition zone (mm $\pm$ SD) ^a				
		<i>C. sinensis</i> EO (10 μ L/disc)	<i>P. lentiscus</i> EO (10 μ L/disc)	<i>E. citriodora</i> EO (10 μ L/disc)	Kanamycin (15 μ g/disc)	Ketoconazole (10 μ g/disc)
<i>Staphylococcus aureus</i> ATCC 29213	G +	17.45 $\pm$ 1.12	21.23 $\pm$ 2.5	16.13 $\pm$ 1.5	10.5 $\pm$ 0.08	Nd
<i>Bacillus cereus</i> (Foodborne isolate)	G +	26.16 $\pm$ 4.04	15.18 $\pm$ 1.15	22.34 $\pm$ 0.6	19.3 $\pm$ 2.38	Nd
<i>Salmonella enterica</i> serotype typhi (Foodborne isolate)	G -	14.05 $\pm$ 0.28	10.11 $\pm$ 0.72	18.06 $\pm$ 1.18	12.46 $\pm$ 2.45	Nd
<i>Escherichia coli</i> ATCC 25922	G -	12.01 $\pm$ 0.5	16.07 $\pm$ 1.53	15.25 $\pm$ 0.03	17.63 $\pm$ 3.1	Nd
<i>Candida albicans</i> (clinical isolate)	Yeast	18.05 $\pm$ 2.01	15.5 $\pm$ 0.75	20.85 $\pm$ 0.67	Nd	18.45 $\pm$ 0.3

^a Ketoconazole and Kanamycin were used as positive control. Results are expressed as means $\pm$ SD, of three independent measurements; Diameter of inhibition zone is including the disc size (6 mm), The disc is loaded with 10 μ L of the EO). Nd: Not determined.

Table 3MIC and MBC value of *E. citriodora*, *P. lentiscus*, *C. sinensis* EOs.

Microorganismes	<i>E. citriodora</i> (% v/v)			<i>P. lentiscus</i> (% v/v)			<i>C. sinensis</i> (% v/v)			Kanamycin (µg/mL)			Ketoconazole (µg/mL)		
	MIC	MBC	MBC/MIC or MFC/MIC	MIC	MBC	MBC/MIC or MFC/MIC	MIC	MBC	MBC/MIC or MFC/MIC	MIC	MBC	MBC/MIC	MIC	MFC	MFC/MIC
<i>S. aureus</i>	0.25	0.25	1.0	1	1	1	0.5	2	4	8.0	8.0	1.0	Nd	Nd	Nd
<i>B. cereus</i>	0.5	1	2.0	1	4	4	0.5	0.5	1	8.0	8.0	1.0	Nd	Nd	Nd
<i>Salmonella enterica</i> serotype <i>typhi</i>	0.25	0.25	1.0	2	2	1	1	1	1	32.0	32.0	1.0	Nd	Nd	Nd
<i>E. coli</i>	0.5	0.5	1.0	4.0	8	2	2	2	1	32.0	64.0	2.0	Nd	Nd	Nd
<i>C. albicans</i>	0.125	0.125	1.0	0.5	0.5	1	0.125	0.125	1	Nd	Nd	Nd	32	128	4

MIC: Minimum inhibitory concentration in % (v/v), MBC: Minimum Bactericidal concentration in % (v/v), *Kanamycin and Ketoconazole (µg/mL) are used as standard drugs. ND: Not determined.

current study. Additionally, the study by Raut and Karuppaiyl [67] demonstrated that *Eucalyptus* spp. oil exhibited strong activity against *S. aureus*, which is consistent with the current study's findings. Similarly, *C. sinensis* oil's antimicrobial activity has been described in previous studies. Within this context, a study by Tao et al. [68] reported that the findings obtained through the disc diffusion and MIC determination techniques demonstrate that the EO exhibits a broad-spectrum of antimicrobial activity against *S. aureus*, *Penicillium chrysogenum*, *B. subtilis*, *E. coli*, and *Saccharomyces cerevisiae*, which is in line with the current study findings. However, it was stated that that *C. sinensis* oil showed weak antibacterial activity against Gram-positive and Gram-negative with inhibition zones ranged between 8.0 and 10 mm [37], which is in contrast to our results.

P. lentiscus EO antimicrobial activity has also been illustrated in the literature. For instance, a study by Alhadad et al. [69] showed that *P. lentiscus* oil exhibited strong activity against *S. aureus*, which is in agreement with the present findings. However, Tabanca et al. and his colleagues [70] indicated that *P. lentiscus* oil did not show any significant activity against *C. albicans*, which contradicts our results. Another study found that it has remarkable antibacterial activity and the study discovered that the antibacterial activity of *P. lentiscus* EO come from a combination of multiple components rather than one particular compound, and different bacteria have varying susceptibilities to the constituents of the oil. Therefore, the antibacterial effect of *P. lentiscus* EO against tested bacteria is likely due to its combination of components, including trace elements [71]. Finally, the variations in antimicrobial findings could be due to several factors, including the type and source of essential oils, the method used for testing antimicrobial activity, and the microorganisms' susceptibility to the oils. Microbial cells depend on their cytoplasmic membrane for the regulation of minor ion movement, which is important for assuring vital cellular functions such as solute transportation, management of turgor pressure, and facilitation of motility [72]. In fact, EOs and their bioactive molecules have demonstrated significant degree of hydrophobicity, allowing them to readily distribute themselves within the lipid-rich domains of bacterial cell membranes and mitochondria [13,73]. This interaction induces the disruption of the membranes' structural integrity, resulting in increased permeability [12]. Previous investigations prove that the monoterpenes, in particular their oxygenated derivatives found in EOs are able to disrupt the integrity of the cell membrane, stop the generation of ATP and thus interrupt the proton motive force. These events leads to the release of the internal components of the cell [74].

3.3. Experimental mixture design

Table 4 lists the mixtures design, which includes various mixtures of *E. citriodora*, *P. lentiscus* and *C. sinensis*, as well as the resulting response of each experiment on five strains, including *E. coli*, *S. aureus*, *S. enterica*, *B. cereus* and *C. albicans* (Fungal strain). The experiments were randomized and each result represents the mean of three replicates.

3.4. Statistical validation of the model

The experimental response data was subjected to statistical analysis to validate the selected model for each studied strain that represents the correlation between the factors and responses. the analysis of variance presented in Table 5 reveals that the $F_{ratio (G/L)}$ computed for each studied responses is greater than the tabular value at the 95% confidence level. For instance, Table 5 shows that the computation of the $F_{ratio (G/L)}$ for *E. coli* (318.02), *S. aureus* (317.66), *S. enterica* (48.92), *B. cereus* (49.45), *C. albicans* (27.68), exhibited a value greater than the tabular value of F at a 95% confidence level. Moreover, since the p -value of the five studied strains is low ($p < 0.05$), we can infer that the main regression effect was statistically-significant.

the coefficient of determination (R^2) was used to verify the confirmation of the models fit to the data. R^2 was equal to 0.99, 0.99, 0.98, 0.99, and 0.99 for *E. coli*, *S. aureus*, *S. enterica*, *B. cereus*, *C. albicans*, respectively. Also, predicted R^2 and adjusted R^2 are also allow to demonstrate the prediction accuracy of the selected models.

These findings were supported by the graph (Fig. 2), which shows a linear curve for the observed values in terms of the predicted

Table 4

Various combinations produced by a selected mixture design and experimental responses obtained for each microbial strains tested.

Number of experiment ^a	<i>E. citriodora</i>	<i>P. lentiscus</i>	<i>C. sinensis</i>	MIC % (v/v) ^b				
				<i>E. coli</i>	<i>S. aureus</i>	<i>S. enterica</i>	<i>B. cereus</i>	<i>C. albicans</i>
1	1	0	0	0.5	0.25	0.25	0.5	0.125
2	0	1	0	4	1	2	1	0.5
3	0	0	1	2	0.5	1	0.5	0.125
4	0.5	0.5	0	2	0.25	1	0.25	0.125
5	0.5	0	0.5	1	0.125	0.25	0.25	0.062
6	0	0.5	0.5	2	0.5	0.5	0.25	0.25
7	0.333	0.333	0.333	0.25	0.125	0.125	0.0625	0.125
8	0.333	0.333	0.333	0.25	0.125	0.125	0.0625	0.125
9	0.333	0.333	0.333	0.25	0.125	0.125	0.0625	0.125
10	0.667	0.167	0.167	0.25	0.0625	0.125	0.125	0.125
11	0.167	0.667	0.167	2	0.5	1	0.25	0.25
12	0.167	0.167	0.667	1	0.25	0.5	0.25	0.062

^a Experiments were carried out after randomization.

^b Each response is the average of three replicates.

Table 5

Variance analysis of studied fitted models.

Model	DF	<i>E. coli</i>				<i>S. aureus</i>				<i>S. enterica</i>				<i>B. cereus</i>				<i>C. albicans</i>			
		SS	MS	F	<i>p</i> -value	SS	MS	F	<i>p</i> -value	SS	MS	F	<i>p</i> -value	SS	MS	F	<i>p</i> -value	SS	MS	F	<i>p</i> -value
G	6	14.48	1.81	318.0209	<0.0001	0.7922	0.1317	317.6666	<0.0001	3.54	0.5906	48.92	0.0003	0.7816	0.1282	49.4581	0.0003 ^a	0.159	0.0257	27.688	0.0011 ^a
L	5	0.0037	0.0012			0.0002	0.0004			0.0604	0.0121			0.0007	0.0025			0.0001	0.0009		
Total	11	14.48				0.7924				3.60				0.7822				0.1591			
R ²		0.99				0.99				0.98				0.99				0.99			
R _{Adj} ²		0.99				0.99				0.96				0.99				0.99			

^a Statistically significant; G: regression; L: residual; R²: coefficient of determination; R_{adj}²: Adjusted R²; Df: degree of freedom; SS: Sum of Square; MS: mean square.

ones.

3.5. Factors effects and the fitted model of all responses

Table 6 summarizes the effects of all factor analyzed, along with their p -values (Observed probability) and t -student statistical values.

The statistically significant coefficients for the response MIC_{E. coli} are those that indicate the activity of the individual EOs (β_1 , β_2 and β_3), followed by the binary interaction coefficient between *P. lentiscus* and *C. sinensis* (β_{23}) and then the ternary term (β_{123}). These findings affirm that antibacterial activity against this bacterial strain depends on all terms except those concerning binary interaction between *P. lentiscus* * *E. citriodora* EOs and *C. sinensis* * *E. citriodora* EOs. The mathematical model adopted for MIC_{E. coli} is represented by the equation below:

$$Y = 0.459X_1 + 4.027X_2 + 2.027X_3 - 3.889X_2X_3 - 33.660X_1X_2X_3 + e \quad (2)$$

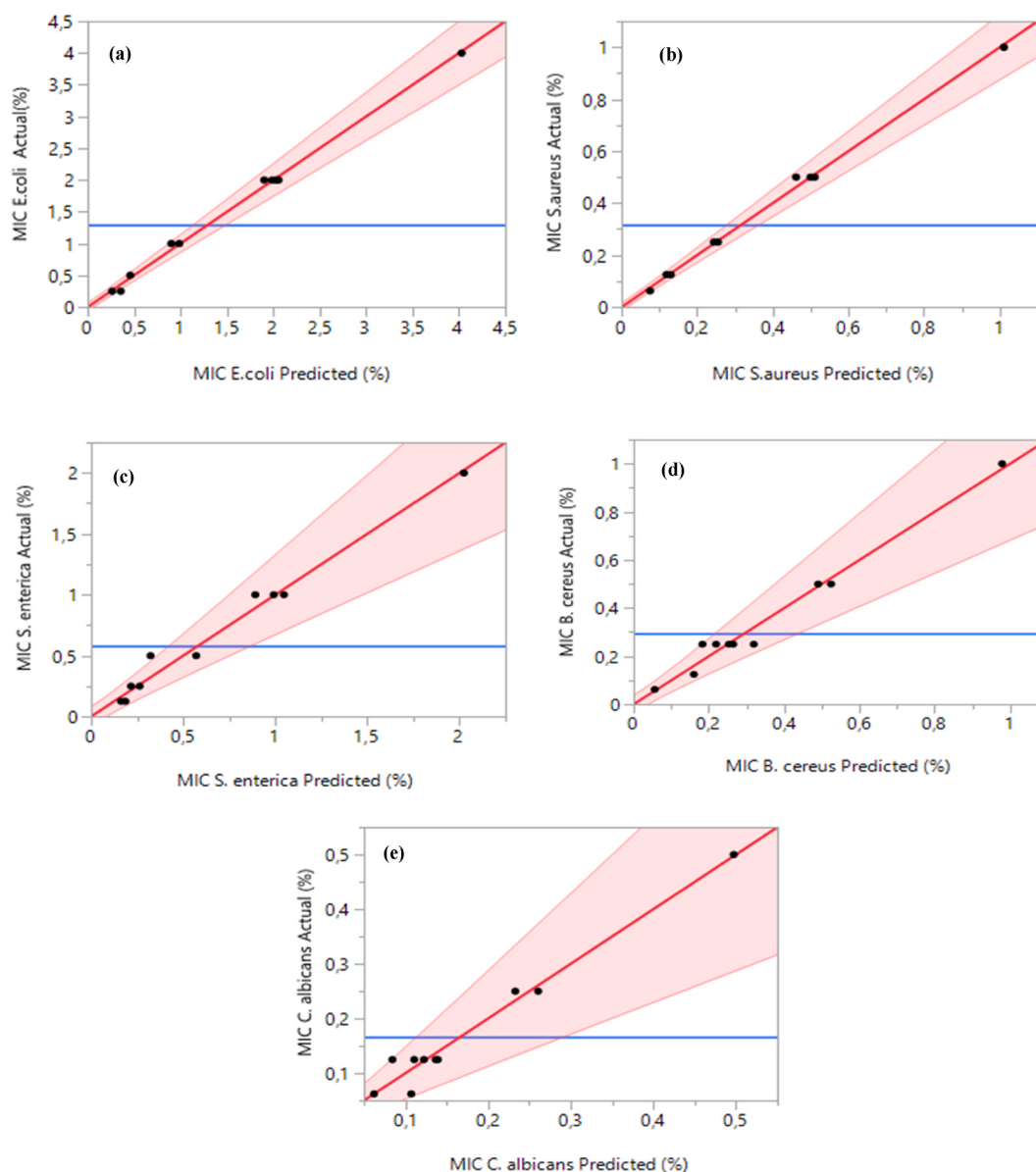


Fig. 2. Curves of the observed values according to the predicted values for the five studied responses (a) *E. coli*, (b) *S. aureus*, (c) *S. enterica*, (d) *B. cereus* and (e) *C. albicans*. The red lines show the curve of actual values of minimum inhibitory concentration (MIC) as a function of those predicted for both strains under study. The blue horizontal lines indicate the mean of the observed values.

Table 6

Estimated regression coefficients of the special cubic model.

Term	Coefficients	MIC <i>E. coli</i>			MIC <i>S. aureus</i>			MIC <i>S. enterica</i>			MIC <i>B. cereus</i>			MIC <i>C. albicans</i>		
		Estimation	t Ratio	P-value	Estimation	t Ratio	P-value	Estimation	t Ratio	P-value	Estimation	t Ratio	P-value	Estimation	t Ratio	P-value
<i>E. citriodora</i>	β_1	0.4595	5.47	0.0028*	0.2440	12.41	<0.0001*	0.2170	2.05	0.0962	0.4903	9.97	0.0002*	0.1393	4.73	0.0052*
<i>P. lentiscus</i>	β_2	4.0277	47.94	<0.0001*	1.0111	51.41	<0.0001*	2.0238	19.07	<0.0001*	0.9789	19.91	<0.0001*	0.4972	16.89	<0.0001*
<i>C. sinensis</i>	β_3	2.0277	24.13	<0.0001*	0.4997	25.41	<0.0001*	1.0465	9.86	0.0002*	0.5244	10.67	0.0001*	0.1108	3.76	0.0131*
<i>E. citriodora</i>	β_{12}	-1.0254	-2.42	0.0598	-1.4895	-15.04	<0.0001*	-0.5181	-0.97	0.3767	-2.0613	-8.33	0.0004*	-0.7267	-4.90	0.0045*
* <i>P. lentiscus</i>																
<i>E. citriodora</i> *	β_{13}	-1.0254	-2.42	0.0598	-1.0122	-10.22	0.0002*	-1.4727	-2.76	0.0400*	-0.9704	-3.92	0.0112*	-0.2516	-1.70	0.1504
<i>C. sinensis</i>																
<i>P. lentiscus</i> *	β_{23}	-3.8890	-9.19	0.0003*	-0.9781	-9.88	0.0002*	-3.8590	-7.22	0.0008*	-1.9931	-8.05	0.0005*	-0.2838	-1.91	0.1138
<i>C. sinensis</i>																
<i>E. citriodora</i> *	β_{123}	-33.6600	-14.63	<0.0001*	-1.8450	-3.42	0.0187*	-7.6500	-2.63	0.0464*	-1.3500	-1.00	0.3622	0.3657	0.45	0.6692
<i>P. lentiscus</i> *																
<i>C. sinensis</i>																

*Statistically significant at $P < 0.05$.

Regarding the response MIC *S. aureus*, the significant terms include all the terms of the adapted mathematical model, namely β_1 , β_2 , β_3 , β_{12} , β_{13} , β_{23} and β_{123} . These findings affirm that the antibacterial effect against *S. aureus* depends on all the interactions between the EOs studied. The following equation illustrates the predictive mathematical model that was retained:

$$Y = 0.244X_1 + 1.011X_2 + 0.499X_3 - 1.489X_1X_2 - 1.012X_1X_3 - 0.978X_2X_3 - 1.845X_1X_2X_3 + \epsilon \quad (3)$$

As for the response MIC *B. cereus*, the statistically significant terms are those that indicate the activity of the individual EOs (β_1 , β_2 and

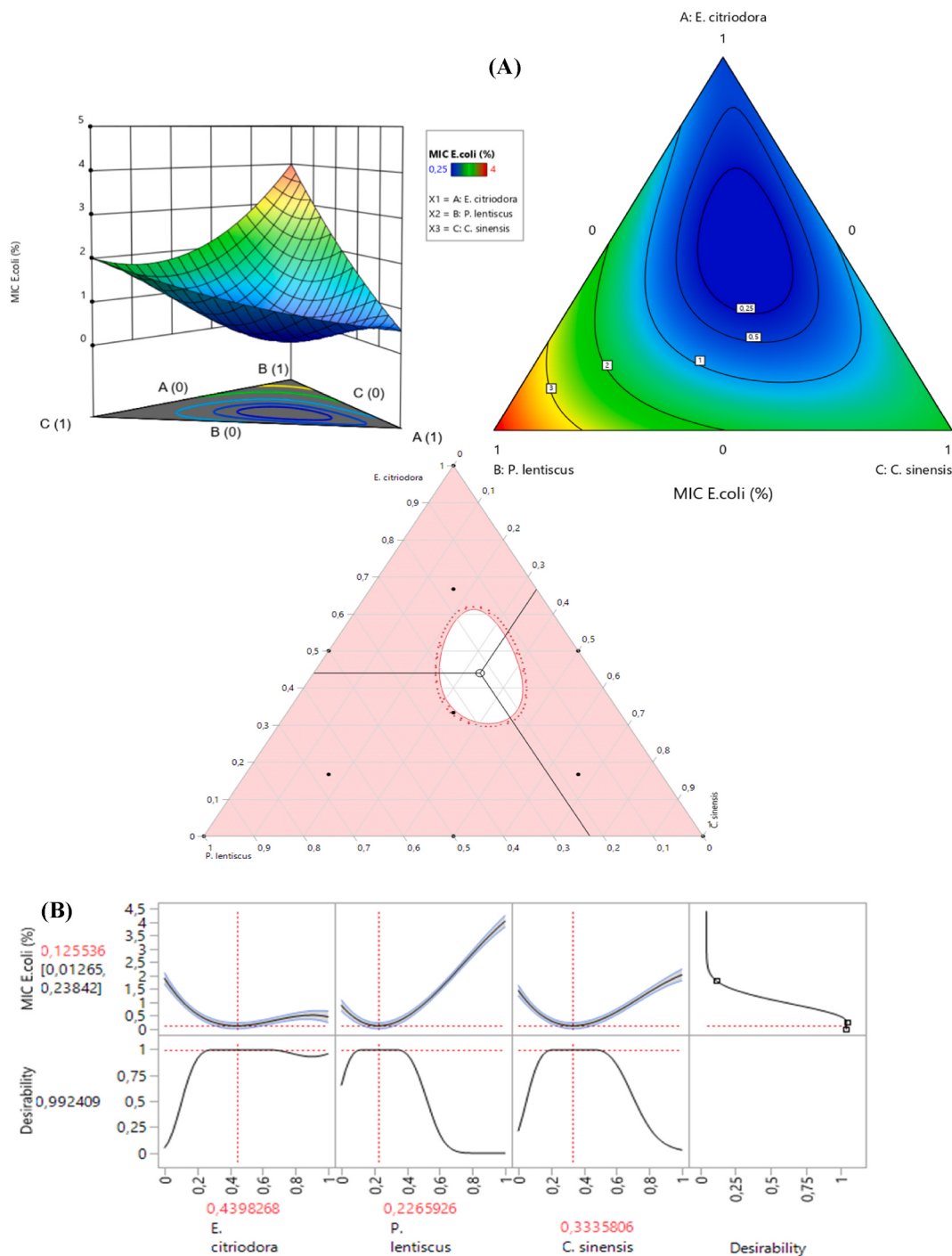


Fig. 3. (A): 2D and 3D mixture plots indicating the optimal compromise area leading to the best values MIC against *E. coli*. (B): Desirability plot displaying the exact proportions of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs that result in the best antibacterial activity against *E. coli* strain.

β_3) and binary interaction terms (β_{12} , β_{13} and β_{23}). This suggests that antibacterial activity against *B. cereus* depends on all interactions, except those expressing the interactions between all three EOs. MIC *B. cereus* response was identified using the subsequent mathematical model:

$$Y = 0.490X_1 + 0.978X_2 + 0.524X_3 - 2.061X_1X_2 + -0.970X_1X_3 - 1.993X_2X_3 + \epsilon \quad (4)$$

Concerning the response MIC *S. enterica*, the linear terms β_2 , β_3 and the binary terms β_{13} , β_{23} , as well as the ternary interaction term β_{123} , were statistically significant. These results prove that the antibacterial effect against this bacterial strain depends on all interactions except those related to the direct effect of *E. citriodora* as well as the interaction between *E. citriodora* and *P. lentiscus*. As a result, the fitted model can be expressed by the following equation.

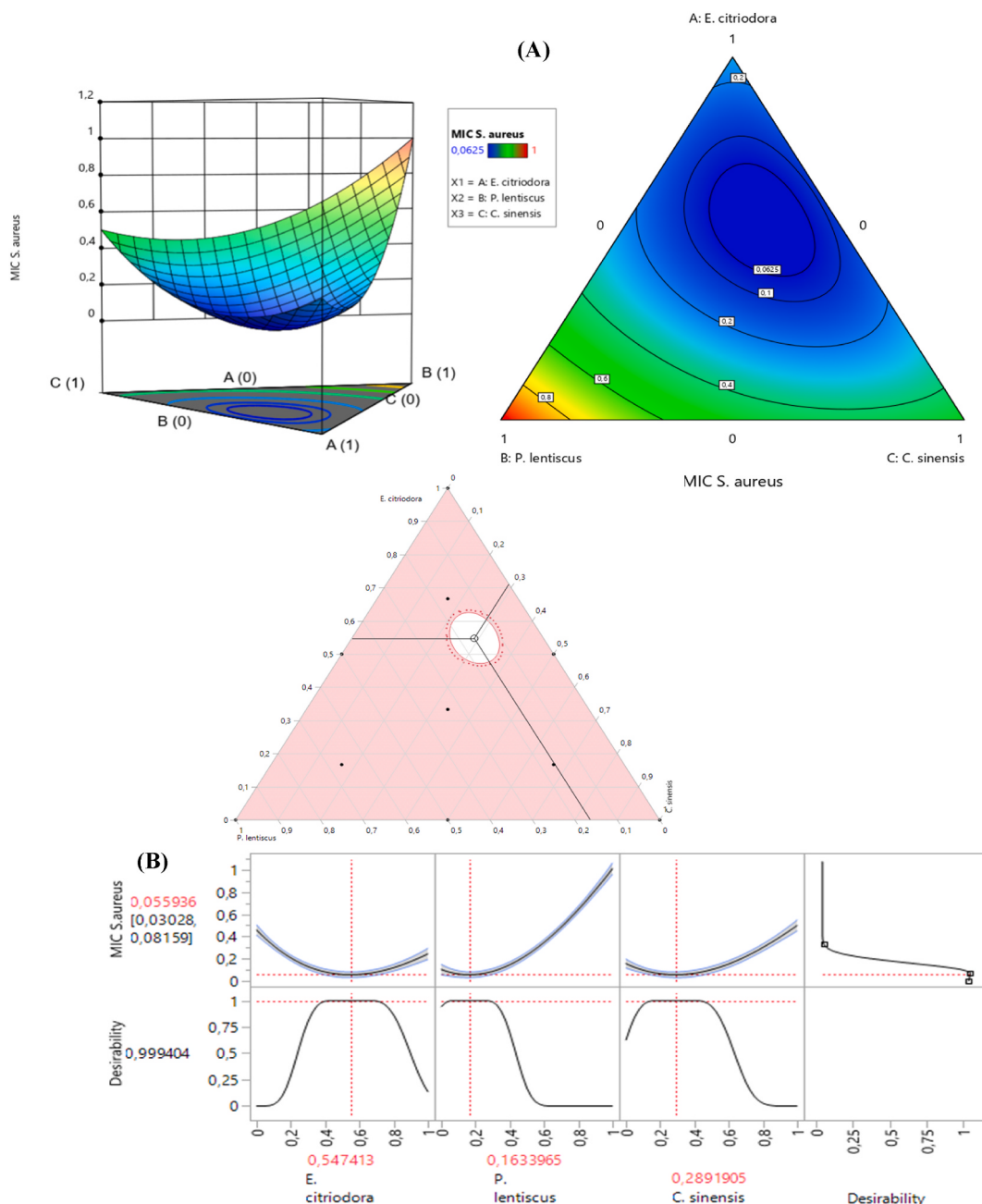


Fig. 4. (A): 2D and 3D mixture plots indicating the optimal compromise area leading to the best values MIC (%) against *S. aureus* strain. (B): Desirability plot displaying the ideal proportions of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs that lead to better antibacterial activity against *S. aureus*.

$$Y = 2.023X_2 + 1.046X_3 - 1.472X_1X_3 - 3.859X_2X_3 - 7.650X_1X_2X_3 + \epsilon \quad (5)$$

As regards the response MIC *C. albicans*, the statistically significant coefficients are those which represent the effects of the individual EOs (β_1 , β_2 and β_3), followed by the coefficient of binary mixture consisting of *E. citriodora* and *P. lentiscus* EOs. These findings indicate that antibacterial activity against *C. albicans* depends on all interactions except those concerning *E. citriodora***C. sinensis* and *P. lentiscus***C. sinensis* and those between the three EOs.

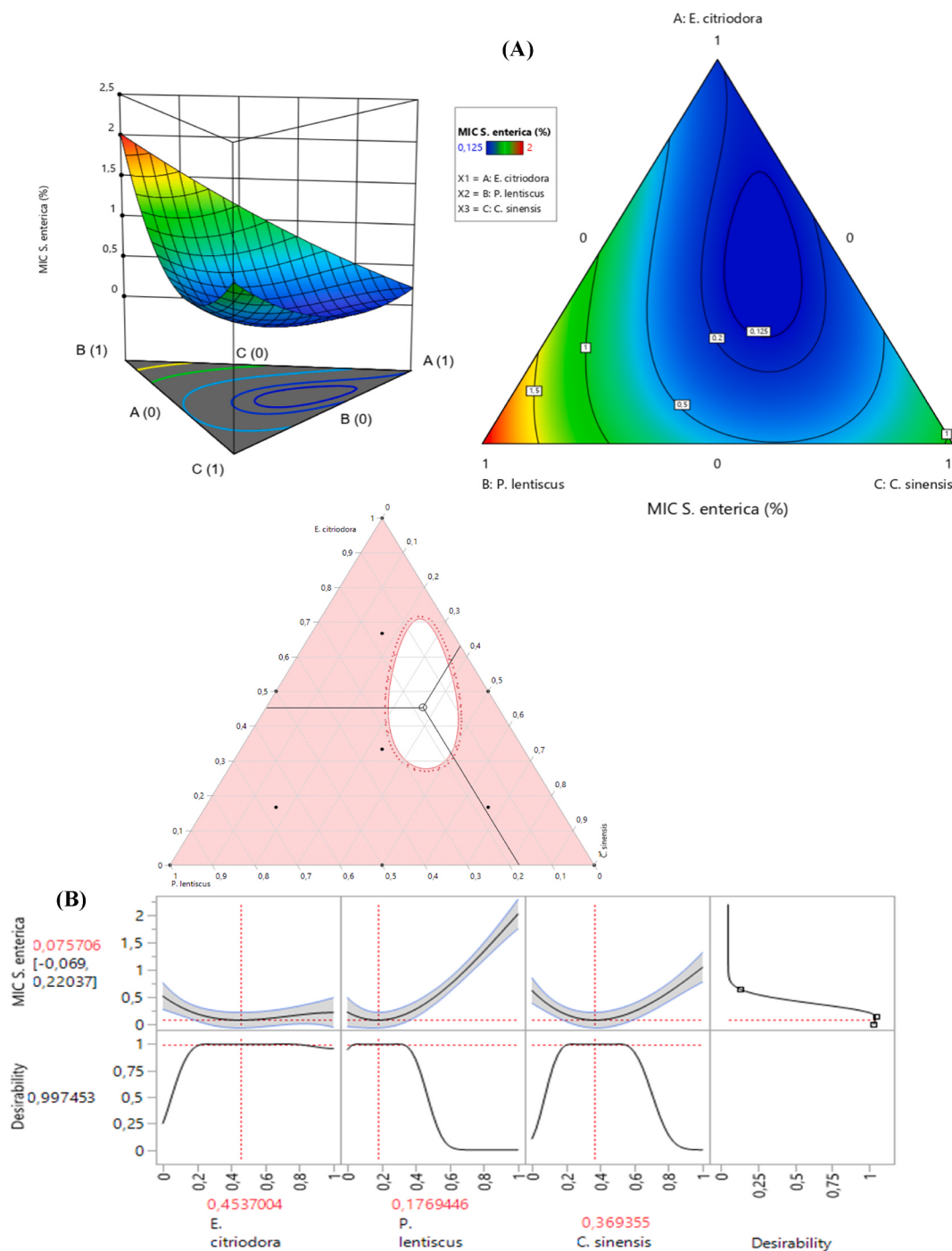


Fig. 5. (A): 2D and 3D mixture plots indicating the optimal compromise area leading to the best values MIC (%) against *S. enterica* strain. (B): Desirability plot displaying the exact proportions of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs that result in the best antibacterial activity against *S. enterica* strain.

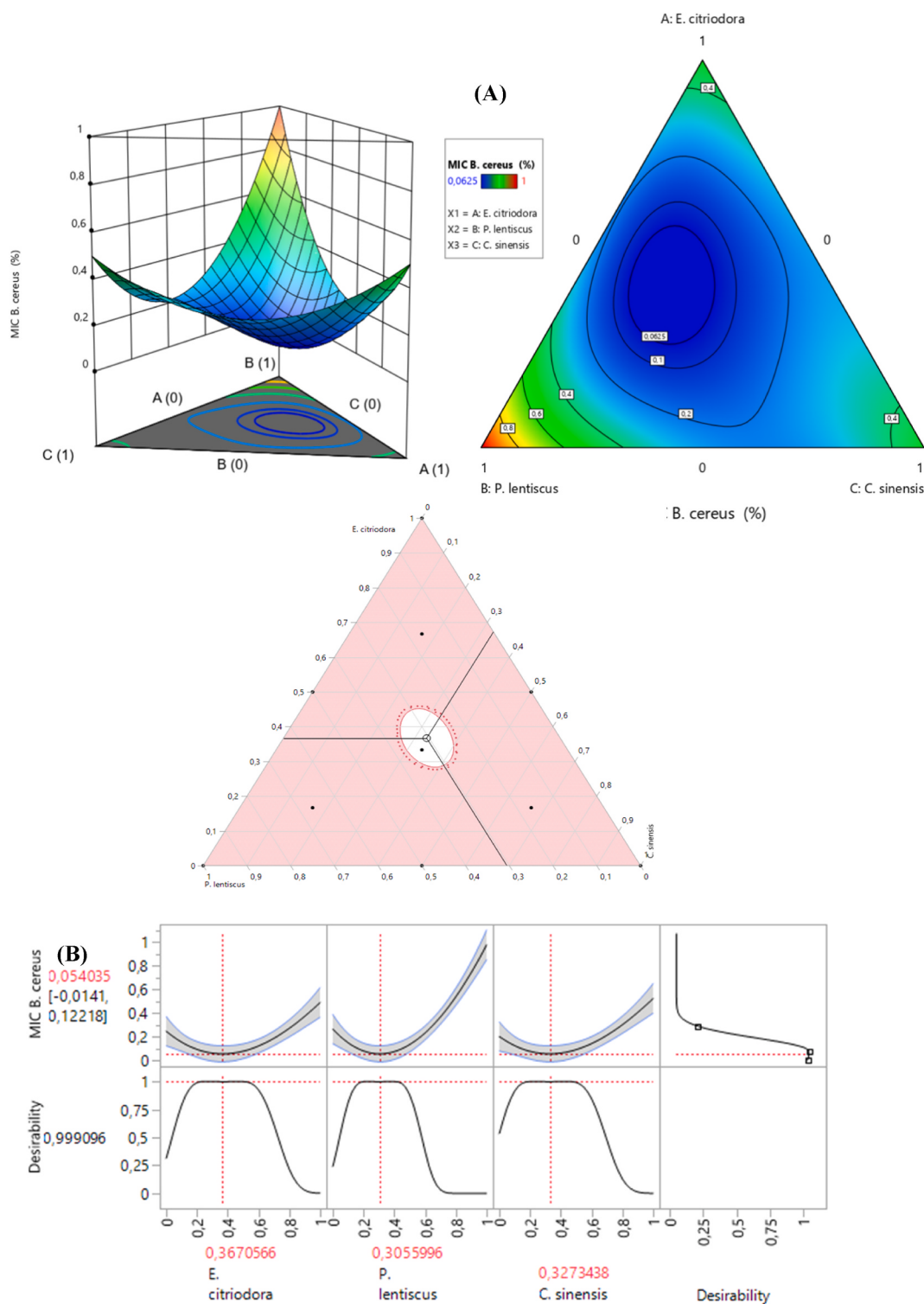


Fig. 6. (A): 2D and 3D mixture plots indicating the optimal compromise area leading to the best values MIC (%) against *B. cereus* strain. (B): Desirability plot displaying the ideal proportions of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs that lead to better antibacterial activity against *B. cereus*.

The mathematical model adopted is presented by the following equation:

$$Y = 0.139X_1 + 0.497X_2 + 0.110X_3 - 0.726X_1X_2 + e \quad (6)$$

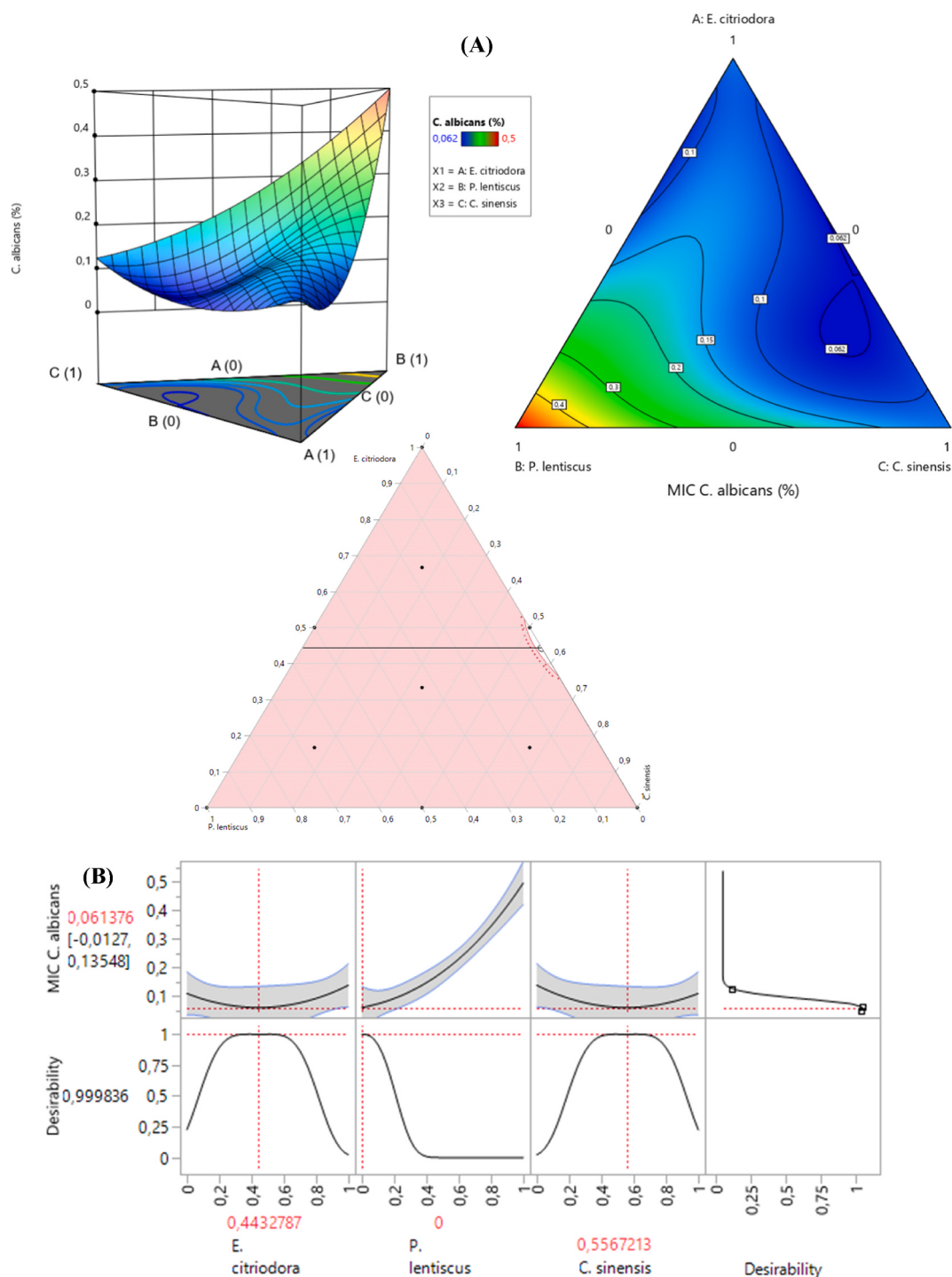


Fig. 7. (A): 2D and 3D mixture plots indicating the optimal compromise area leading to the best values MIC (%) against *C. albicans* strain. (B): Desirability plot displaying the exact proportions of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs that result in the best antibacterial activity against *C. albicans* strain.

3.6. Formulation optimization and desirability study

The optimization process entails combining the three EOs in ways that can produce better results than those obtained from the individual oils. Therefore, the purpose of this study is to identify the optimal formulation of the three EOs in order to attain the lowest possible MIC value. While conducting experiments, we noticed that the lowest MIC values obtained were 0.250%, 0.0625%, 0.125%, 0.0625 and 0.0625% for *E. coli*, *S. aureus*, *S. enterica*, *B. cereus* and *C. albicans*, respectively. Therefore, any MIC value that is equal or lower to these values will be considered the desired value for optimization.

This study highlights that the optimal formulation of the three studied EOs had stronger antimicrobial effects against the tested strains, compared to the individual EOs. This positive interaction is also illustrated in Fig. 8, where the optimal area in the mixing zone is located precisely in the center of the triangle. The 2D and 3D mixture plot indicated the interactions between each independent variable utilized in the mixture. In the 2D and 3D mixture plots, the dark blue colored area pointed to lower MIC values and greater microbial efficiency, while the green to red tint represent medium to higher MIC values. Thus, augmented simplex-centroid design optimized the proportion of each active compound in a mixture, to create an optimal formulation, which was characterized by its strong antimicrobial activity.

3.6.1. Efficacy of the EOs formulation against *E. coli*

The MIC value for *E. coli* obtained from various EOs combinations ranged from 0.25 to 4% (Table 4). Fig. 3A presents the contour and surface plots of the response MIC_{*E. coli*} obtained using various mixtures of the three EOs. As a compromise against *E. coli*, the MIC was set at 0.25%. From the 2D and 3D mixture plot (Fig. 3A), we can deduce that a mixture composed of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs is necessary to attain a MIC of 0.25%. Furthermore, the desirability graph (Fig. 3B) confirmed these findings and shows that the best achievable value is equal to 0.125% v/v with desirability of 99%. To achieve this value, a mixture of 43% *E. citriodora*, 22% *P. lentiscus*, and 33% *C. sinensis* should be ensured.

3.6.2. Efficacy of the EOs formulation against *S. aureus*

The MIC value for the response MIC_{*S. aureus*} ranged from 0.0625 to 1% (Table 4). The displayed illustration in the 2D and 3D mixture plot (Fig. 4A) provide valuable insight into the optimal compromise area, indicating that achieving the desired MIC (0.0625%) requires a mixture consisting of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs. Furthermore, the desirability function supports these results and suggests that a mixture consisting of 54% *E. citriodora*, 16% *P. lentiscus* and 28% *C. sinensis* EOs can give a MIC of 0.055% with a desirability of 99% (Fig. 4 B).

3.6.3. Efficacy of the EOs formulation against *S. enterica*

Fig. 5, shows the 2D and 3D mixture plot realized for MIC_{*S. enterica*}, obtained using a different mixture of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs.

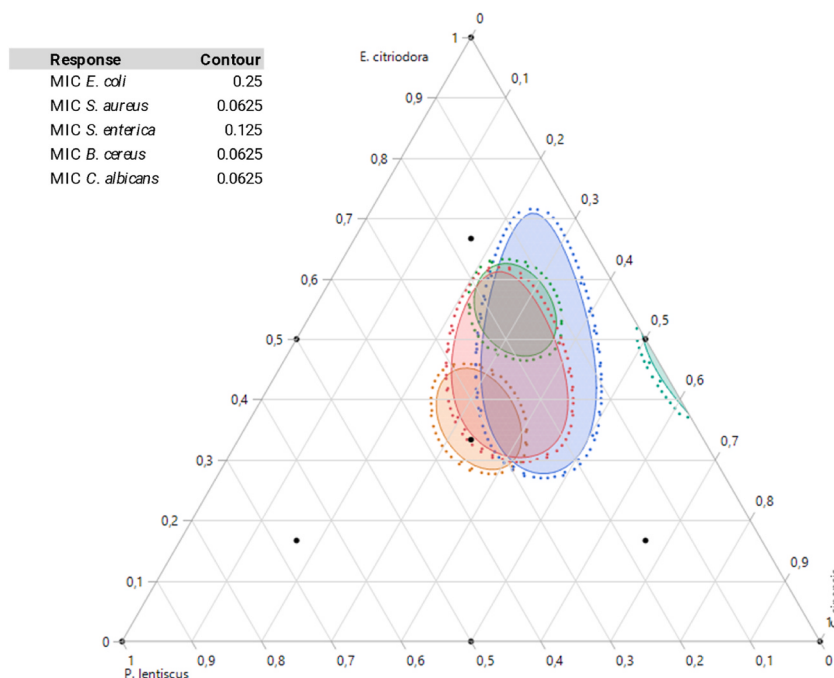


Fig. 8. Mixture contour plot illustrating the optimal combination zone between *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs against all microbial strains tested.

The MIC value for *S. enterica* obtained from various EOs combinations ranged from 0.125 to 1% (Table 4). The illustration in the 2D and 3D mixture plot (Fig. 5 A) indicate that a mixture of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs is required to achieve a value of approximately 0.125%. Furthermore, the desirability test in Fig. 5B indicates that a maximum MIC value of 0.0757% can be attained with a desirability of 99% by realizing a mixture comprising about 45% *E. citriodora*, 17% *P. lentiscus* and 36% *C. sinensis*.

3.6.4. Efficacy of the EOs formulation against *B. cereus*

The recorded values for the MIC_{*B. cereus*} response was in the range of 0.0625–1%. The contour and surface plots for this response (Fig. 6A), allows us to explore the various activities associated with different proportions of the three EOs studied. A MIC of 0.0625% was determined as a compromise against *B. cereus*. From the 2D and 3D mixture plot, we can conclude that a mixture of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs is necessary to achieve this MIC value. Furthermore, the desirability test (Fig. 6 B) confirms these results and indicates that the best attainable value is equal to 0.054% with a compromise percentage of 99% by realizing a mixture composed of 36% of *E. citriodora*, 30% of *P. lentiscus* and 32% of *C. sinensis*.

3.6.5. Efficacy of the EOs formulation against *C. albicans*

The MIC *C. albicans* value relating to the various experiments ranged from 0.062 to 0.5% (Table 4). Thanks to Fig. 7 A, we can conclude that the optimal compromise area corresponding to the desired MIC (0.062%) requires the use of a mixture composed essentially of *E. citriodora* and *C. sinensis* EOs. Consequently, *P. lentiscus* EOs should be fixed in its low percentage 0%. These findings were confirmed by the desirability test (Fig. 7 B), which shows that we can achieve a MIC of 0.061% with a compromise percentage of 99% by ensuring a mixture composed of 44% of *E. citriodora* and 55% *C. sinensis* EOs, binary mixture.

Researchers commonly use this particular type of mixture design model to investigate the possible interactions among several active compounds and to predict the ideal combination [75–80]. Ouardghi et al. [77] optimized the concentrations of *Origanum majorana* L., *Thymus serpyllum* L., and *Origanum compactum* Benth. EOs using a mixture design methodology. The ideal mixture predicted against *E. coli* corresponded to 75% *O. compactum* and 25% *O. majorana*, while the predicted optimal mixture against *S. aureus* and *B. subtilis* was composed of 30% *O. majorana*, 42% *T. serpyllum*, and 28% *O. compactum* EOs. Chraïbi et al. [79] used a mixture design to study the potential synergistic effects between *Ormenis mixta* (L.) Dumort., *Mentha pulegium* L., and *Mentha piperita* L., EOs against *Candida tropicalis*, *E. coli* and *S. aureus*. Recently, they showed also the role of mixture design in the optimization of the antibacterial effect of EOs [78].

3.7. Synergy between three studied EOs

Mixture plot (Fig. 8) displayed the optimal combination zone between *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs against all studied strains. The locations of five responses in the combined mixture plot indicates a potential correlation among them. The compromise zone sought between the three EOs proportions to achieve the desired MIC requires a mixture composed of *E. citriodora*, *P. lentiscus* and *C. sinensis* EOs for *E. coli*, *S. aureus*, *S. enterica*, *B. cereus* strains, whereas a mixture of *E. citriodora* and *C. sinensis* EOs is required for *C. albicans* (Fig. 8). This mixture of EOs are mainly ascribed to the presence of oxygenated monoterpenes such as 1,8-cineole, β -citronellol, citronellal; and monoterpene hydrocarbons, including α -limonene, careen-3, β -myrcene. Each of the mentioned molecules have multiple sites of action on microbial cells [49,80].

The oxygenated terpenoids, such as monoterpenes and sesquiterpenes, are the major antimicrobial components compared to the terpene hydrocarbons, which do not contain hydroxyl groups (-OH) in their chemical structure [49]. Additionally, the interaction between major and minor compounds may act synergistically to have a significant effect on microorganisms [81]. For example, some hydrocarbon monoterpenes such as myrcene, terpinene, and limonene are ineffective antimicrobial agents when used alone, but show significant antimicrobial effects when combined with phenols such as carvacrol [78]. In fact, these molecules can further swell bacterial cell membranes, making it easier for thymol and carvacrol to penetrate bacterial cell membranes, thus achieving a synergistic effect [49,78]. In addition, it has been reported that combinations of limonene/1,8-cineole, carvacrol/myrcene, 1,8-cineole/thymol and 1,8-cineole/carvacrol exhibit a synergistic antimicrobial effect [82–85]. Furthermore, Burt et al. [12] suggested that a synergistic effect or potentiating influence between two EOs can be attributed to interactions between minor components that may play a critical role in the antibacterial activities of the mixtures.

As a conclusion, it can be said that a mixture two or three EOs would increase their antimicrobial activity and provide effective microbial control. It remains to be noted that the four optimal mixtures predicted against *E. coli*, *S. aureus*, *S. enterica* and *B. cereus* exist in the same area of the experimental field which means that they require almost identical optimal mixtures.

4. Conclusion

Here, we investigated the formulation of three chemically-characterized EOs extracted from common plants, namely sweet orange, lentisk and lemon eucalyptus using mixture design methodology. The chemical identification revealed the abundance of α -limonene (14.27%) and careen-3 (14.11%) in lentisk EO, while citronellal (39.40%) and α -limonene (87.22%) were the principal volatile components detected in lemon eucalyptus and sweet orange, respectively. Moreover, the antimicrobial potential of these EOs varied according to the proportions of each component in the mixture and the target microbe. Minimal inhibitory concentrations were significantly reduced from 0.25%<MIC<4%–0.0625%<MIC<2% using the combinations of sweet orange, lentisk and lemon eucalyptus EOs. This effect could be related to synergistic effect between active volatile compounds of the combined EOs. The best combinations predicted on *C. albicans*, *S. aureus*, *E. coli*, *S. enterica* and *B. cereus* correspond to 44%/55%/0%, 54%/16%/28%, 43%/

22%/33%, 45%/17%/36% and 36%/30%/32% of lemon eucalyptus, lentisk and sweet orange EOs, respectively. These combinations may serve as an alternative to commercial drugs and chemical preservatives, which are becoming more and more ineffective to contract several microbes causing serious infectious and alteration of food products.

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Author contribution statement

Naoufal El Hachlafi, Hanae Naceiri Mrabti and Abdelhakim Bouyahya: Conceived and designed the experiments; Wrote the paper; Formal analysis; Contributed reagents, materials, analysis tools or data. Samiah Hamad Al-Mijalli: Wrote the paper; Performed the experiments; Mohamed Jeddi: Analyzed and interpreted the data; Wrote the paper; Performed the experiments. Emad M. Abdallah: Wrote the paper; Performed the experiments. Hamza Assaggaf, Ahmed Qasem and Bodour S. Rajab: Contributed reagents, materials, analysis tools or data. Learn-Han Lee: Contributed materials; wrote the paper; Khang Wen Goh, Long Chiau Ming: wrote the paper, Conceived and designed the experiments.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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