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Research Article

Application of Simplex-Centroid Design Methodologies to Optimize the Anti-bacterial and Anti-candidal Activity of the Mixture of *Mentha pulegium*, *Pituranthos chloranthus* and *Thymus algeriensis* Essential Oils

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ABSTRACT

This work aims to study the antimicrobial activities of the essential oils of M. *pulegium*, *P. chloranthus* and *T. algeriensis* and to identify the antagonistic or synergistic impacts of their combinations. Antimicrobial activities were analyzed by disc diffusion and microdilution against six foodborne microbial strains (*Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa* and Candida *albicans*). That's why, to predicate the optimal mix, we are using the *Augmented Simplex Centroid Design*. The chemical composition shows that the main compound of M. *pulegium* were pulegone (50.31%) and menthone (26.92%). For *P. chloranthus* EO, The major constituents were dillapiole (13.56%) and pregnane (8.47%) and the main products of *T. algeriensis* EO are Camphor (14.06%), β-Phellandrene (13.71%) and α-pinene (8.55%). The antimicrobial activity of the three studied EOs confirms the highest antibacterial activity of *T. algeriensis* and *P. chloranthus* EO against a lower antibacterial activity of M. *pulegium* EO. Moreover, the response surface analysis showed significant synergistic effects in some binary and ternary mixtures. The optimal mixture predicted against C. *albicans* corresponded to 12%, 35% and 53% of M. *pulegium*, *P. chloranthus and T. algeriensis EOs*, respectively. While the optimal mixture predicted against *S. aureus* was composed by *P. chloranthus* and *T. algeriensis* essential oils at 84% and 16%, respectively. Similarly, this binary mixture, between *P. chloranthus and T. algeriensis*, presented optimal antimicrobial activity against *P. aeruginosa* composed by essential oils at 20% and 80%, respectively. Generally, the combination between 19% M. *pulegium*, 41% *P. chloranthus and* 40% *T. algeriensis* consisting the optimal ternary mixture Eos. Our findings show that by the use of mixture design we can predict antibacterial interaction of essential oils. Therefore, it can be used as a natural antimicrobial agent and a food additives.

Keywords: Mixture design; Antibacterial activity; Essential oils; *Mentha pulegium*; *Pituranthos chloranthus*; *Thymus algeriensis*

INTRODUCTION

For many years there has been intense interest in essential oils as source of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infections and as natural food preservatives [1]. In fact, the essential oils represent an inexpensive source of natural antibacterial substances for use in pathogenic systems to prevent the growth of bacteria and extend the shelf life of the processed food [2]. Furthermore, enhancing food preservation and getting a balance between the sensory acceptability and antimicrobial efficacy

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is actually possible by the addition of small amounts of natural preservatives such as EOs. The *Mentha pulegium L.* (Lamiaceae) is widespread in America and thrives in Western, Southern and Central Europe, Asia, Iran, Arab countries and Ethiopia.

Mentha pulegium is widely used in folk medicine in many cultures, aromatherapy and cosmetics [3]. The flowering aerial parts of this plant are traditionally used for their antimicrobial, expectorant, carminative and antispasmodics in the treatment of colds, bronchitis, tuberculosis, sinusitis, cholera, food poisoning, flatulence skin diseases, abortifacient and intestinal colic [4]. They strengthen the entire nervous system, stimulating diffusible and also a diffusible sedative; mint provides eminent services against nervousness and various nervous manifestations [5]. *M. pulegium* essential oil can be used as an antibiotic, a bio-insecticide and an organic food preservative, therefore a natural replacement for harmful synthesized chemicals. The essential oils of *M. pulegium* characterized by its richness in menthol, menthone and pulegone.

The genus *Pituranthos* has more than twenty species, some of which are specific to North Africa [6]. *Pituranthos species* are often found in arid or desert regions. They are widespread in central and southern Tunisia. The genus *Pituranthos* has several therapeutic effects. Indeed, the species *P. triradiatus* and *P. tartuosus*, are used by the Bedouin population against stomach pains, intestinal parasites or as a menstrual regulator in women. Oils obtained from the stems and seeds of *Pituranthos scoparius* are widely used as a remedy against rheumatism and fever [7]. The species *Pituranthos chloranthus* is used, in poultices on the head, against the headache. In Morocco, the aerial parts are mixed with the ashes to flavour the meat [8]. It is also used in seasonings [9]. *P. chloranthus* stems have traditionally been used as straw by farmers to dry figs and grapes. In southern Tunisia, a tuft of *P. chloranthus* was traditionally suspended from the surface of water to disinfect underground rainwater storage tanks used for beverages [10]. There are many phytochemical studies on the *Pituranthos chloranthus* species, in particular, on its essential oils. Indeed, have demonstrated antimicrobial activity *of P. chloranthus* essential oils against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*. The results of Yangui et al. clearly indicate the effective bactericidal and fungicidal action of essential oils of Tunisian *P. chloranthus* [7].

Thyme is considered as one of the most valuable spices and food preservatives in the food industry. *T. algeriensis* is the most pervasive North African species, endemic to Morocco, Tunisia, Algeria and Libya. It is is largely used in traditional medicine, in respiratory and digestive tube disorders and against abortion. Essential oils have been investigated in several studies where the scope was the chemical analysis of these compounds and their biological activities against several bacteria, yeast and fungi [11-13].

Goals of this work were to optimize the antibacterial and anticandidal Activity of the mixture of *M. pulegium*, *P. chloranthus* and *T. algeriensis* essential oils utilizing the Simplex- centroid Design.

MATERIALS AND METHODS

Plant materials

The aerial parts of *T. algeriensis* and *P. chloranthus* were collected from Jabal Sagoufta, Qafsah (at the southwestern of Tunisia; latitude 34°30'0" (N); longitude 9°18'0" (E), altitude 567 m) in Avril. Plant populations of *M. pulegium*, was collected from Sousse, in central Tunisia, in May. The harvested plants were identified by Professor Fethia Harzallah-Skhiri (high institute of biotechnology of Monastir, Tunisia). The plant material was dried in the open air, in the dark and at room temperature for 2 weeks until constant weight. Moisture content was assessed by constant weight at 105°C and was 5.0 ± 0.5%, 4.47 ± 0.3 and 5.23 ± 0.41 of *M. pulegium*, *P. chloranthus* and *T. algeriensis*, respectively.

Extraction of the essential oil

Dried samples of the selected plants (100 g and 800 mL of water, ten times) were subjected to hydro-distillation for Clevenger in accordance with European Pharmacopoeia method [14].

The mixture was heated to boiling temperature and the liberated steams crossed up the column and passed out of the condenser in a liquid state. The obtained oils were separated completely from water without adding any solvent. The yield of the oil (based on dry plant weight) measured and stored in a freezer at 4°C in dark glass bottles until used.

In order to eliminate any trace of water in the Essential Oils (EO), the recovered distillate is stored in the freezer (-20°C) for 24 hours. The water phase is frozen and the essential oil is separated, measured and stored in freezer at 4°C in dark glass bottles until used EO yield (%) was measured using the following formula [15]:

EO yield (%) = Mass of EO obtained (g)/Mass of dry matter (g) × 100

Essential oil analysis

The analyze was carried out using GC 6890N and 5975B MS Agilent model, equipped with an Agilent Technologies capillary HP-5MS column (30 m × 0.25 mm i.d.) form of fused silica HP type -1 (0.25 µ film thickness) and an electron collision ionisation (70 eV ionization energy). The temperature of the injector starts from 35°C and increases 5°C/min to 250°C. The convayer gas was Helium used at 1 mL/min flow rate.

Identification of components was assigned by matching their mass spectra with Wiley and NIST library data standards of the main components and comparing their Kovats Retention Indices (KRI) with reference from the literature. The components concentrations were obtained by semi-quantification by peak area integration from GC peaks and by applying the correction factors [16].

The simplex centroid design method: 3-factors mixture design and triangular surface analyses were performed to optimize the antibacterial activity of mixture of three plants essential oils. The simplex-centroid design is composed with seven experiments. It is constructed to form a triangle with data points located at each corner, the three midpoints on each side, as well as the center (Figure 1).

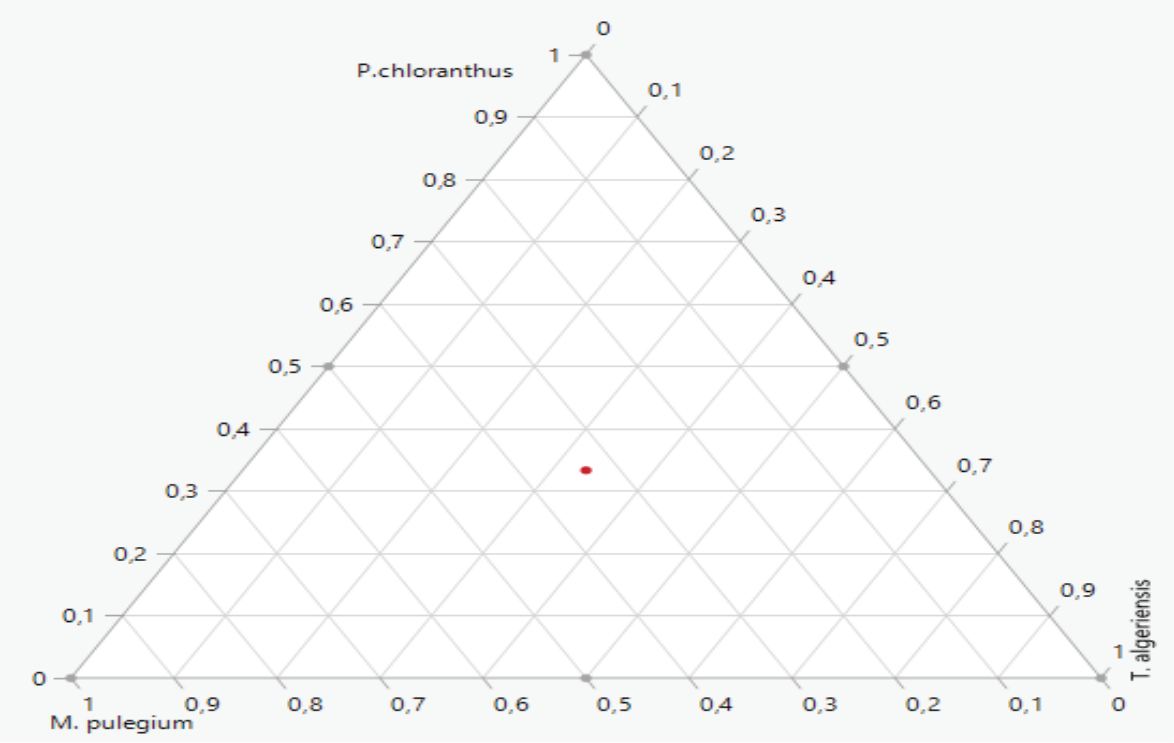


Figure 1: Experimental points for the augmented Simplex-centroid design.

Mathematical models of mixing plans: The mathematical model applied to the mixing plans takes into account the fundamental constraint of mixing. The polynomials used have particularities that we will indicate.

First degree model: It is assumed that the variations in response are proportional to the composition of three-component mixture**.**

Taking into account the fundamental constraint of mixtures:

$x1 + x2 + x3 = 1$

The model takes the following form:

$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3$

Second degree model: The second degree mathematical model includes first degree terms, quadratic terms and square terms. For a three-component mixture, we have:

$Y = \beta 1\ x_1 + \beta_2\ x_2 + \beta_3\ x_3 + \beta_{12}\ x_1\ x_2 + \beta_{13}\ x_1\ x_3 + \beta_{23}\ x_2 x_3$

Third degree model: The simplified model, corresponding to a mixture of three components is as follows:

$Y = \beta_1\ x_1 + \beta_2\ x_2 + \beta_3\ x_3 + \beta_{12}\ x_1\ x_2 + \beta_{13}\ x_1\ x_3 + \beta_{23}\ x_2 x_3 + \beta_{123}\ x_1\ x_2\ x_3$

Where, y is the response function of experimental data, x_1, x_2 and x_3 independent variables which correspond to the percentage of *M. pulegium, P. chloranthus and T. algeriensis* respectively, in the mixture.

$\beta_i = Y_i$

$\beta_{ij} = 4\ Y_{ij} - 2(Y_i + Y_j)$

$\beta\iota_{jk} = 27Y_{ijk} - 12(Y_{12} + Y_{13} + Y_{23}) + 3(Y_1 + Y_2 + Y_3)$

Preparation of mixtures of essential oils: The Simplex-centroid design has been used to prepare the mixtures of Eos. It composed of seven experiments with two replications at the center point. The obtained solutions were vortexed for about three min. Each solution has been diluted with DMSO (1:10 v/v) and vortexed again. The final volume of each eight mixtures was 6000 µL.

Antibacterial and anti-candidal activity

Microorganisms: Antibacterial and antifungal activity was evaluated on different microorganisms provided by the Laboratory of Transmissible Diseases and Biological Active Substances (Faculty of Pharmacy of Monastir). Six microbial foodborne strains (5 bacteria and Candida) were used: *Bacillus cereus* (ATCC 6633), *Salmonella enterica* (CIP 8039), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 30031).

The micro-organism was grown in sterile broth medium for 24 h. Then 0.1 mL of each culture was mixed with 9.9 ml of fresh broth medium for 18 h.

Preparation of the culture medium

Preparation of Mueller Hinton medium: 38 grams of powder were added to 1 liter Distilled water and mixed thoroughly. The medium was boiling for 1-2 minutes to dissolve the constituents. Then, pH of the solution is adjusted to 7.3 and autoclaved at 120°C for 25 min.

Preparation of nutrient broth: 38 g of powder are dissolved in one liter of distilled water. The flask is placed in a boiling water bath until homogeneous, liquid, transparent solution is obtained. The solution is then poured into tubes, after which the latter have to be autoclaved at 120°C for 25 min.

Agar diffusion tests: In order to evaluate the antimicrobial activity of essential oils, we used the agar diffusion method of the National Committee for Clinical Laboratory Standards [17]. The bacteria and C. *albicans* were prepared from colonies of less than 24 h in Petri dishes. A single colony was mixed in 15 mL of the broth and

then incubated for 3 to 5 hours at 35° C. then, Mueller- Hinton agar was inoculated with 100 µL of 10^6 CFU mL^{-1} bacterial cultures.

The plates were dried for 15 min. Then, Sterilized paper discs (6 mm) were impregnated with 10 µL of different EOs. After 30 min of diffusion at laboratory temperature, the Petri dishes were then incubated at 37°C for 18 to 24 hours. After the incubation, the antibacterial activity was expressed as the diameter of the inhibition zones (DIZ) produced and measured in mm unit.

The tests were repeated twice, the Standard commercial antibiotics (Gentamicin) are used as a positive control.

The results were ranked as follows: not sensitive for zone diameters equal to 8 mm or below; sensitive for zone diameters between 8 and 14 mm, very sensitive for zone diameters between 14 and 20 mm and extremely sensitive for zone diameters equal or larger than 20 mm [18].

Minimum Inhibitory Concentration (MIC): The MIC is the lowest sample concentration capable to inhibit bacterial visible growth [19]. The MIC, MBC and MFC were determined according to the protocol [20]. Inocula of each bacterial strain were prepared in physiological water from a young culture. The eight samples of essential oils were dissolved in 10% dimethyl sulfoxide (DMSO). The suspensions were adjusted to a concentration of 10^6 bacteria/mL. MICs for the EOs were performed on a 96-wellplate:

- In column 1, we are deposed, 160 µL of Mueller-Hinton, 20 µL of the microbial suspension, 10 µL Gentamicin as positive control and 10 µL of resazurin as growth indicator, which is initially blue and turns pink in case of cell growth.
- In column 2, we are deposed, 170 µL of Mueller-Hinton, 20 µL of the microbial suspension and 10 µL of resazurin as negative control.
- In colomn 3, we are deposited a 200 µL of EOS mixture and dilution series of factor 2 was carried out while taking 100 µL of Column No. 3 and adding them in column No. 4 and so on to column 12. The last 100 µL of the wells of column 12 are discarded.
- From the third to the twelfth column, we added 70 µL culture medium 20 µL of the microbial suspension and 10 µL of resazurin.
- The plates were incubated at 37°C for 24 hours. The last well before the change of color in pink indicates the MIC.

Minimum Bactericidal Concentration (MBC)/Minimum Fungicide Concentration (MFC): The main steps in determining the MBC and MFC are as follows: 10 µL are taken from the well corresponding to the MIC and the one before. The specimens were then streaked (5 cm) onto the agar. The seeded Petri dish was subsequently incubated at 37°C for 24 hours. Finally, the absence of colonies of bacteria means that the corresponding concentration is that of the MBC. The CMB/CMI ratio allowed us to determine the bactericidal and bacteriostatic properties of the essential oil studied. When this ratio is greater than 4, the essential oil has a bacteriostatic power, and bactericidal when it is less than or equal to 4 [21].

Statistical analysis

The statistical significance of each equation is evaluated with Tukey's test at $P<0.05$. The ternary surface response diagram, polynomial model and principal component analysis were generated, was accomplished using JMP software.

RESULTS AND DISCUSSION

Essential oil yields

Essential oils obtained from the aerial parts (stems and leaves) of *M. pulegium, T. algeriensis and P. chloranthus* yielded 2.4%, 1.2% and 0.89% (w/w), respectively.

M. pulegium could be considered as an appreciated source of EO and the obtained yields were higher than other ones collected from the north region of Tunisia (1.5%) and 1.84%. In Algeria, The extraction yield of EO from dried leaves of *M. pulegium* cultivated is about 1.45 ± 0.01%. However the result is similar to that of Morocco oil (2.33) and 2.7%. In Algeria, the average yield in leaves essential oils of *T. algeriensis* was significantly high (1.52-2.02%). The essential oil from the aerial parts of *T. algeriensis*, obtained by hydrodistillation, was obtained in a yield of 2.8 ± 0.2%, w/w [22]. The leaves yielded recorded for Tunisian *T. algeriensis* can be considered high compared to other Thymus species that are used industrially as a source of essential oils [12].

P. chloranthus EO yields from the fresh and dry herb collected from the Matmata's mountainous chain in southern Tunisia are 1.6% and 0.9%, respectively [10].

Chemical composition

Twenty-five components accounting for 98.17% of the total amount of Tunisian *M. pulegium* EO were that represented approximately for Table 1. The EO is characterized with a high amount of oxygenated monoterpenes (82.78%). Main compound were pulegone (50.31%) and menthone (26.92%) followed by Palmitinic acid (7.32%). Therefore, pulegone is the oil chemotype. This has been confirmed by Abdelli et al. Hajlaoui et al. Bouchra, et al. They show that the percentage of this component varies between 25% to 92%. These results were in accordance with other studies. In fact, algerian study showed the richness of *M. pulegium* in pulegone (44.27%), menthone (19.05%) and piperitone (10.44%). Pulegone is also the major compound of Moroccan oil (69.8%) followed by piperitenone (3.1%). Moroccan study shows that The *M. pulegium* EO contains the pulegone (40.98%) and the menthone (21.164%) as major constituents. However, the major components of a fresh plant essential oil, were collected from the region of Beja (north of Tunisia) were menthol (39.2%), 1,8-cineole (17.1%), menthone (12.6%) and pulegone (11.7%). The chemical composition of Iranian oil is especially monoterpene: piperitone 38%, Menthone 39% [23]. Other compounds were poorly represented such as Isopulegone 2.89 and Steric acid 1.46 (Table 1). The total monoterpene hydrocarbons are 3.37%. The oxygenated sesquiterpenes and the sesquiterpene hydrocarbons are 1.69 and 1.43, respectively.

For *P. chloranthus* EO, The major constituents were dillapiole (13.56%) and pregnane (8.47%). The essential oil contains mainly monoterpene hydrocarbons (40.07%) and oxygenated monoterpenes (17.13%). In literature, there is difference on composition between *P. chloranthus* oils. This suggests that there are different chemotypes of *P. chloranthus* in Tunisia. For exemple,

Table 1: Chemical composition, KI, retention time and percentage composition of the essential oils extracted from *M. pulegium*, *P. chloranthus* and *T. algeriensis*

No	Identification	KI	R_t (min)	Composition-Percentages (%)		
				M. pulegium	*P. chloranthus*	*T. algeriensis*
1	Tricyclene	926	6.04	-	-	0.34
2	α-Thujene	931	6.23	-	2.82	0.28
3	α-Pinene	940	6.52	0.96	7.43	8.55
4	Camphene	953	6.73	-	0.11	4.05
5	Verbenene	967	6.86	-	-	0.53
6	β-Pinene	971	7.44	0.20	-	4.74
7	β-Phellandrene	975	6.76	-	-	-
8	Sabinene	978	7.53	-	16.39	-
9	β-Myrcene	980	7.87	0.12	1.02	0.86
10	α-Phellandrene	1005	8.25	-	1.45	0.30
11	3-Carene	1011	8.35	0.16	3.40	-
12	α-Terpinene	1016	8.60	-	1.45	1.22
13	Cymene	1020	8.83	-	1.40	-
14	d-Limonene	1031	8.88	0.79	-	-
15	β-Phellandrene	1026	8.92	-	1.16	-
16	1,8-Cineole	1031	9.02	-	-	13.71
17	α-Ocimene	1035	9.21	-	-	-
18	β-Ocimene	1040	9.23	-	0.37	1.07
19	γ-Terpinene	1058	9.80	-	2.28	1.95
20	4-Thujanol	1072	10.05	-	1.06	0.96
21	α-terpinolene	1081	10.61	-	0.79	1.08
22	Linalool	1098	11.03	-	-	2.68
23	1- terpineol	1105	11.61	-	0.83	-
24	Nopinone	1122	12.02	-	-	-
25	Camphor	1136	12.27	-	-	14.06
26	dl-Menthone	1148	12.58	26.92	2.66	-
27	Pinocarvone	1157	12.73	-	-	0.97
28	Borneol	1161	12.94	-	-	1.25
29	Isopulegone	1167	13.15	2.89	-	-
30	4-Terpineol	1171	13.31	-	7.32	4.22
31	Cuminol	1177	13.51	-	-	
32	Menthol	1180	13.52	0.42	-	-
33	α-Terpineol	1189	13.62	-	1.00	3.07
34	Myrtenal	1192	13.68	-	-	-
35	Myrtenol	1212	13.94	-	-	0.86
36	Verbenone	1228	14.10	-	-	1.30
37	Carveol	1235	14.44	-	-	0.92
38	O-Methylthymol	1244	14.74	-	-	0.28
39	Cuminaldehyde	1255	14.99	-	-	0.22
40	Carvone	1263	15.05	-	-	0.37
41	Pulegone	1271	15.17	50.31	3.95	-
42	linalyl acetate	1279	15.33	-	-	1.28
43	piperitone	1283	15.39	0.49	-	-
44	Carane	1291	15.88	1.05	-	-
45	Camphane	1293	15.88	-	-	-
46	α-Fenchyl acetate	1311	16.13	-	-	3.36
47	Trans-Carane	1342	16.75	0.76	-	-
48	Carvacrol	1356	16.95	-	0.31	
49	3-Terpinolenone	1361	17.69	0.70	-	-
50	α-Terpinene	1365	17.82	0.38	-	-

51	α-Ionone	1367	17.83	-	-	-
52	geranyl propanoate	1371	18.23	-	-	0.20
53	α-Copaene	1378	18.49	-	0.10	0.15
54	β-Bourbonene	1384	18.72	-	-	0.26
55	β-Elemene	1391	18.91	0.18	-	0.15
56	α-Gurjunene	1415	19.33	-	-	0.27
57	Methyleugenol	1423	19.35	-	1.11	-
58	β-Caryophyllene	1442	19.60	0.15	0.31	0.35
59	α-Bergamotene	1449	20.00	-	0.12	-
60	α –humulene	1451	20.46	0.34	-	0.04
61	β-Farnesene	1443	20.53	-	0.24	-
62	Aromadendrene	1456	20.63	-	-	0.27
63	Germacrene-D	1468	21.17	-	0.64	0.43
64	Bicyclogermacrene	1471	21.50	-	-	0.45
65	Mint furanone	1480	21.66	0.75	-	
66	α-Farnesene	1505	21.81	-	0.20	-
67	α-Amorphene	1512	21.94	0.25	-	0.22
68	Δ-Cadinene	1525	22.15	-	-	0.56
69	L-calamenene	1527	22.16	0.68	-	-
70	Myristicin	1532	22.39	-	2.40	-
71	α-Calacorene	1539	22.62	-	-	0.06
72	Hedycaryol	1541	22.85	-	-	-
73	Elemol	1546	22.87	-	-	0.65
74	Elemicin	1554	23.08	-	0.40	-
75	Palustrol	1562	23.24	-	-	0.15
76	Caryophyllene oxide	1576	23.59	0.34	-	2.23
77	Spathulenol	1585	23.60	-	0.89	
78	Veridiflorol	1614	23.90	-	-	3.31
79	Globulol	1623	24.09	-	-	0.38
80	Adipol	1643	24.53	-	-	0.14
81	Dillapiole	1649	24.55	0.12	13.56	-
82	Cadina-1,4-diene	1658	24.65	0.43	-	-
83	β-Maaliene	1672	24.72	-	-	0.13
84	β-Eudesmol	1693	25.20	-	1.47	0.47
85	t-Muurolol	1703	25.25	-	-	0.21
86	β-Himachalene	1716	25.38	-	-	0.73
87	Azulol	1736	25.69	-	-	0.24
88	14-Norcadin-5-en-4-one	1771	26.00	-	-	0.16
89	Butylphthalide	1821	27.24	-	0.37	-
90	Palmitinic acid	1876	27.92	7.32	-	1.39
91	2-Pentadecanone	1902	29.21	-		-
92	Steric acid	1928	30.73	1.46	-	-
93	Dodecanamide	1943	31.93	-		
94	13-Epimanoyl oxide	1954	32.11	-	-	-
95	Manoyl oxide	1968	32.17	-	0.58	-
96	Anthracene	2015	32.72	-	-	-
97	Dehydroabietane	2026	33.08	-	0.33	-
98	Oleic acid	2093	34.17	-	-	0.40
99	Hexadecane	2112	34.31	-	-	
100	Steric acid	2134	34.41	-	-	0.74
101	Tetracosane	2226	36.17	-	-	
	(17E)-Pregna-5,17-dien-3-ol	2251	36.80	-	-	-
102	Hexacosane	2356	38.41	-	-	-

103	γ.-Sitosterol	2485	41.51	-	-	-
104	Hentriacontane	2524	44.44	-	-	-
105	Pentyl acetate	2562	45.17	-	-	-
106	14B-Pregnane	2584	46.66	-	8.47	0.19
107	Cetyl vinyl ether	2588	46.67	-	-	-
108	Nonacosane	2896	46. 96	-	-	0.15
109	Hexatriacontane	2902	46.97	-	-	-
110	Capnellene	2913	47.00	-	2.67	-
111	Clionasterol	2928	47.24	-	-	
112	Sitostenone	2934	47.39	-	-	-
113	Spongesterol	2938	47.40	-	2.27	
114	Friedelin	2972	47.56	-	-	-
115	3-Ethylstyrene	2981	47.59	-	-	-
116	Cycloartenol	2993	47.64	-	1.44	-
117	Epifriedelinol	3134	48.25	-	-	-
118	Stigmastan-3,5-dien	3165	48.64	-	-	0.11
	Total monoterpene hydrocarbons			3.37	40.07	24.67
	Total oxygenated monoterpenes			82.78	17.13	46.15
	Total sesquiterpene hydrocarbons			1.43	9.66	5.17
	Total Oxygenated sesquiterpenes			1.69	8.26	10.85
	Total identified compounds			98.17	94.77	89.11

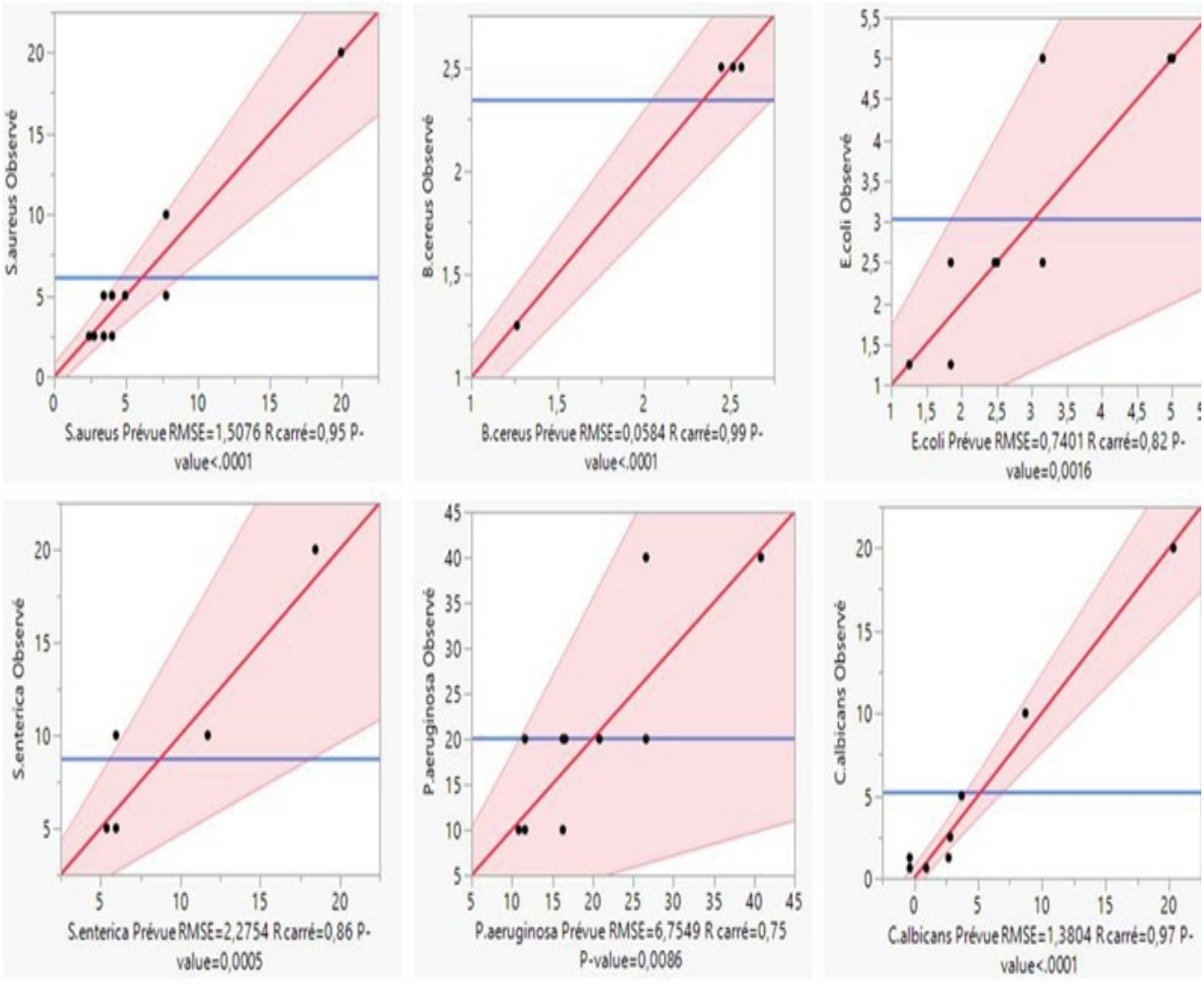


Figure 2: Curve of the observed values in terms of the predicted values of each bacterial strains tested.

the main constituents of the EO obtained from *P. chloranthus* collected in Southern Tunisia were found to be α-pinene (32.5%), β-phellandrene (13.9%) and α-phellandrene (7.8%). EO of *P. chloranthus* collected from Sfax present also main constituent (terpinen-4-ol (30.3%)) which is different from other regions in Tunisia and other countries. A comparison between these studies showed the variability of volatile compound. The variation in the obtained results is certainly attributed to the difference in geographical locations and growth conditions that affect the chemical composition of Eos.

At last, according to the results on Table 1, the constituents of the essential oil of *T. algeriensis* is more than 60 compounds with an overall percentage of 89.11%. The chemical composition is particular and characteristic of the essential oil. This table shows that the essential oil contains mainly oxygenated compound (46.15%) and monoterpene hydrocarbons (24.67%).

The main products are Camphor (14.06%), β-Phellandrene (13.71%) and α-pinene (8.55%). Similarly, Mehalaine et al. identified camphor (13.62%) as the main constituent of *T. algeriensis* EO. However, the results in the literature indicate that the essential oils of *Thymus algeriensis* collected in Tunisia, Algeria and Morocco have a high level of carvacrol. In fact, the main compound of essential oils of *T. algeriensis* cutivated from three Tunisian region (Korbous, Jdidi Jebel Montain and Hammem Sousse) were caryophylleneoxide (18.5-25.3%), veridiflorol (tr-39.7%), α-pinene (2.7-15.2%), 1,8-cineole (1.2-12.8%), p-eugenol (tr-15.8%), geraniol (tr-7.1%). In Algeria, Salah Bendjabeur (2018) showed that the main constituents were carvacrol (43.2%), γ-terpinene (14.8%), p-cymene (18.7%), and thymol (5.6%). In Marroco, The essential oil was characterized by high amounts of Geranyl acetate (80.8%). The other major components were Geraniol (7.3%) and trans-Caryophyllene (2.4%). But, work of Hamdani showed that borneol (28%), camphene (20.9%) and camphor (15.7%) were the major compounds. *T. algeriensis* EO collected from Libya thymol was the main constituent (38.5%) fol-lowed by p-cymene, terpinene, bornyl acetate and borneol (8.9%, 7.1%, 7.0% and 6.0%, respectively).

Evaluation of antibacterial activity

Single antibacterial effect: The study of the antimicrobial activity of EO by the disc diffusion method shows that, with the exception of *P. aeruginosa*, *M. pulegium* EO has a bacteriostatic activity against all the strains tested. Table 2 shows that Gram-positive; *S. aureus* (14 ± 1 mm) and *B. subtilis* (13 ± 2 mm) bacteria are very sensitive to essential oils. For Gram-negative bacteria and Yeasts, *M. pulegium's* EO has significant inhibitory effects against *E. coli* (11 ± 0.8 mm), *S. enteritidis* (10 ± 1.5 mm) and Candida *albicans* (11 ± 1.5 mm).

As shown in Table 2, CMI values confirm those obtained previously by Aromatogram method. The strains tested are sensitive to *M. pulegium* EO at different concentrations from 2.5 to 20 µL/mL. The lowest MIC value (2.5 µL/mL) was observed in B. cereus.

Similarly, Abdelli et al. show that *M. pulegium's* EO is active against bacteria Gram négative such as, *Bordetella bronchiseptica*, *Escherichia coli*, *Pasteurella multocida*, *Salmonella enteritidis* and *S. gallinarum pullorum*. They show also that bacteria Gram positive are extremely sensitive to this EO in particular *S. aureus* (23 ± 1 mm) and B. subtilis (24 ± 0 mm). Another study carried out by Ait-Ouazzou et al. indicate that *M. pulegium's* EO inhibited the growth of *E. coli* by causing an inhibition zone with a diameter of 12.6 ± 0.5 mm. in like manner, the results found by Hajlaoui et al. showed that *M. pulegium* EO has a strong anti-microbial activities in particular against Gram-positive bacteria with inhibition zones in the order of 10-31 mm [24]. However, the study by Boukhatem et al. (2014) shows that yeasts are more resistant than the pathogenic bacteria tested [25].

The potential antimicrobial activity of this *M. pulegium* EO is explained with the high level of oxygenated monoterpenes (82.78%) in particular pulegone and menthone. However, other trace components could increase the antimicrobial activity. In addition to that, it is possible to have a synergistic and antagonistic interaction between the components. Several researchers have studied the mode of action of EO. They have shown that antimicrobial activity is caused by the action of terpenes in enzymatic systems related to energy production and in the synthesis of structural components of microbial cells [26]. In fact, EO crosses the cell membrane, interacting with enzymes and proteins in the H+/ATPase pumping membrane, producing a proton flow that induces cell death. In addition, terpenes can affect the permeability and other functions of cell membranes. Indeed, EO crosses cell membranes, enters the cell and interacts with critical intracellular sites [27].

Table 2: Antimicrobial and anticandidal activity of *Mentha pulegium*, *Pituranthos chloranthus* and *Thymus algeriensis* essential oils.

Microbial strains	*M. pulegium*			*P. chloranthus*			*T. algeriensis*		
	Inhibition zone (mm)	MIC (µL/mL)	MBC (µL/mL)	Inhibition zone (mm)	MIC (µL/mL)	MBC (µL/mL)	Inhibition zone (mm)	MIC (µL/mL)	MBC (µL/mL)
Bacteria Gram+									
S. aureus	11 ± 1	20	40	19 ± 1.75	2.5	5	15 ± 2	5	5
B. cereus	13 ± 2	2.5	5	14 ± 1.25	1.25	5	12 ± 1.5	2.5	5
Bacteria Gram-									
E. coli	11 ± 0.8	5	10	16 ± 0.75	2.5	2.5	13 ± 1.25	1.25	5
S. enterica	10 ± 1.5	5	5	12 ± 1.2	5	5	11 ± 1.4	5	5
P. aeruginosa	<8	-	-	9 ± 1	20	40	9 ± 1	10	20
Yeasts									
C. albicans	11 ± 1.5	20	20	17 ± 1.2	2.5	2.5	19 ± 1.5	0.625	2.5

Means values ± SD of triplicate determination

Table 3: Original components of the design matrix and experimental responses (MICs) obtained for each bacteria.

	Original components of the design matrix				Experimental responses (MICs)				
	M. pulegium	*P. chloranthus*	*T. algeriensis*	*S. aureus*	*B. cereus*	*E. coli*	*S. enterica*	*P. aeruginosa*	*C. albicans*
1	0.000	0.000	1.000	5.000	2.500	1.250	5.000	10.000	0.625
2	0.000	0.000	1.000	5.000	2.500	1.250	5.000	10.000	0.625
3	0.000	0.500	0.500	2.500	2.500	2.500	20.000	10.000	1.25
4	0.000	0.500	0.500	2.500	2.500	1.250	20.000	20.000	0.625
5	0.000	1.000	0.000	2.500	1.250	2.500	5.000	20.000	2.5
6	0.000	1.000	0.000	2.500	1.250	2.500	5.000	20.000	2.5
7	0.333	0.333	0.333	5.000	2.500	10.000	10.000	10.000	1.25
8	0.333	0.333	0.333	5.000	2.500	10.000	10.000	10.000	1.25
9	0.333	0.333	0.333	2.500	2.500	10.000	10.000	10.000	1.25
10	0.333	0.333	0.333	2.500	2.500	10.000	10.000	20.000	1.25
11	0.500	0.000	0.500	2.500	2.500	10.000	10.000	20.000	5.000
12	0.500	0.000	0.500	5.000	2.500	5.000	5.000	20.000	5.000
13	0.500	0.500	0.000	10.000	2.500	2.500	10.000	20.000	10.000
14	0.500	0.500	0.000	5.000	2.500	5.000	5.000	40.000	10.000
15	1.000	0.000	0.000	20.000	2.500	5.000	5.000	40.000	20.000
16	1.000	0.000	0.000	20.000	2.500	5.000	5.000	40.000	20.000

The results of the evaluation of the antimicrobial activities of *P. chloranthus* and *T. algeriensis* EOs are presented in Table 2. This result exhibited a potent antibacterial activity against all tested bacteria and fungi. They indicate that Gram negative bacteria were more susceptible to the antimicrobial properties of essential oils (inhibition zones in the order of 9-14 mm) than Gram positive bacteria and yeasts (inhibition zones in the order of 14-19 mm).

Accurately, MICs/MFC and MBSs values for *P. chloranthus* essential oil against *S. aureus*, *B. cereus*, *E. coli*, *S. enterica* and *C. albicans* colonies ranged from 1.25 µL/mL to 5 µL/mL; for *T. algeriensis* essential oil these values were varied from 0.625 µL/mL to 5 µL/mL. However, P. aeruginosa were found to be less sensitive to the tested EOs and tend to display higher MIC values (20 µL/mL and 10 µL/mL for *P. chloranthus* and *T. algeriensis* Eos, respectivily) and higher MBC (ranged from 40 to 20 µL/mL). The activity of essential oils correlates with their chemical function. Indeed, the biological activity of an essential oil is related to its chemical composition, the functional groups of the majority compound. Minority compounds also play an important role in the activity of essential oils and appear to act in synergy with the main compounds) [28].

The high antimicrobial activity *P. chloranthus* and *T. algeriensis* EOs is explained by the presence of high amounts of oxygenated monoterpenes (17.13 and 46.15%, respectively) and monoterpene hydrocarbons (40.07 and 24.67, respectivily). Indeed, Cox et al. show that monoterpenes are capable of affecting cellular integrity, leading to inhibition of respiration and altered permeability [29].

Similarly, the results of Yangui et al. indicate the effective bactericidal and fungicidal action of essential oils of Tunisian *P. chloranthus* where the antimicrobial effect against four microorganisms : *P. aeru*ginosa, *E. coli*, *S. aureus* and *E. hirae*, as well as on yeasts: *C. albicans* and *Aspergillus niger*. In addition, Neffati, in 2009 reported good activity bacteriostatic of the essential oil of *P. chloranthus* against all Gram-positive bacteria studied, in particular *S. aureus* and *L. monocytogene* [30].

The antibacterial and antifungal activities of *T. algeriensis* EOs have studied by Ali, which is in agreement with our obtained results. They showed moderate antibacterial and antifungal activities with growth zone ranged from 13.6 to19.4 mm. The highest MIC value was detected against *P. aeruginosa*, while the lowest was observed against B. cereus. A moderate inhibitory concentration was against *L. monocytogenes* and *E. coli* (MICs=3.5-5 µL/mL). In conclusion, essential oil is a complex mixture of different chemical components, thus it is difficult to reduce the antimicrobial effect of the total essential oil to one or more active ingredients. In addition, other work shows that minor components, as well as a possible interaction between substances, may affect antimicrobial activities. The high antibacterial and antifungal activities of Eos suggests the possibility of using this plant as a natural antimicrobial preservative in the food and pharmaceutical systems.

Figure 3 shows ternary contour plots of the responses (MICs) for each studied bacteria. Optimal design regions for the antibacterial effect of EOs mixture are the colourless areas. Figure 3b indicates that the CMI contour values increased toward the *P. chloranthus* where minimum CMI contour could be seen. Figure 3e and Figure 3f indicate that the CMI contour values increased toward *T. algeriensis*. Moreover, Figure 3d shows that the CMI contour values increased toward *M. pulegium*; however, for *E. coli* (Figure 3c) strain the lowest CMI values were at *T. algeriensis-P. chloranthus* edge.

Similarly, the isoresponses curves (Figure 4) shows the compromise areas between the components. In fact, the colourless areas in the Figures 4a, 4b, 4e and 4f indicate CMI values less than 2.5, Figure 4d the isoreponse curves of CMI values less than 5 delimit the colourless zone. Whereas, the colourless areas in the Figure 4c indicate CMI values less than 15. Besides, the exact optimal combination with a percentage of compromise was found by use of the "Desirability" function. The exact optimum setting has shown in Figure 5. The minimum CMI values against *E. coli* (Figure 5a), *B. cereus* (Figure 5b) and *S. enterica* (Figure 5c) are obtained

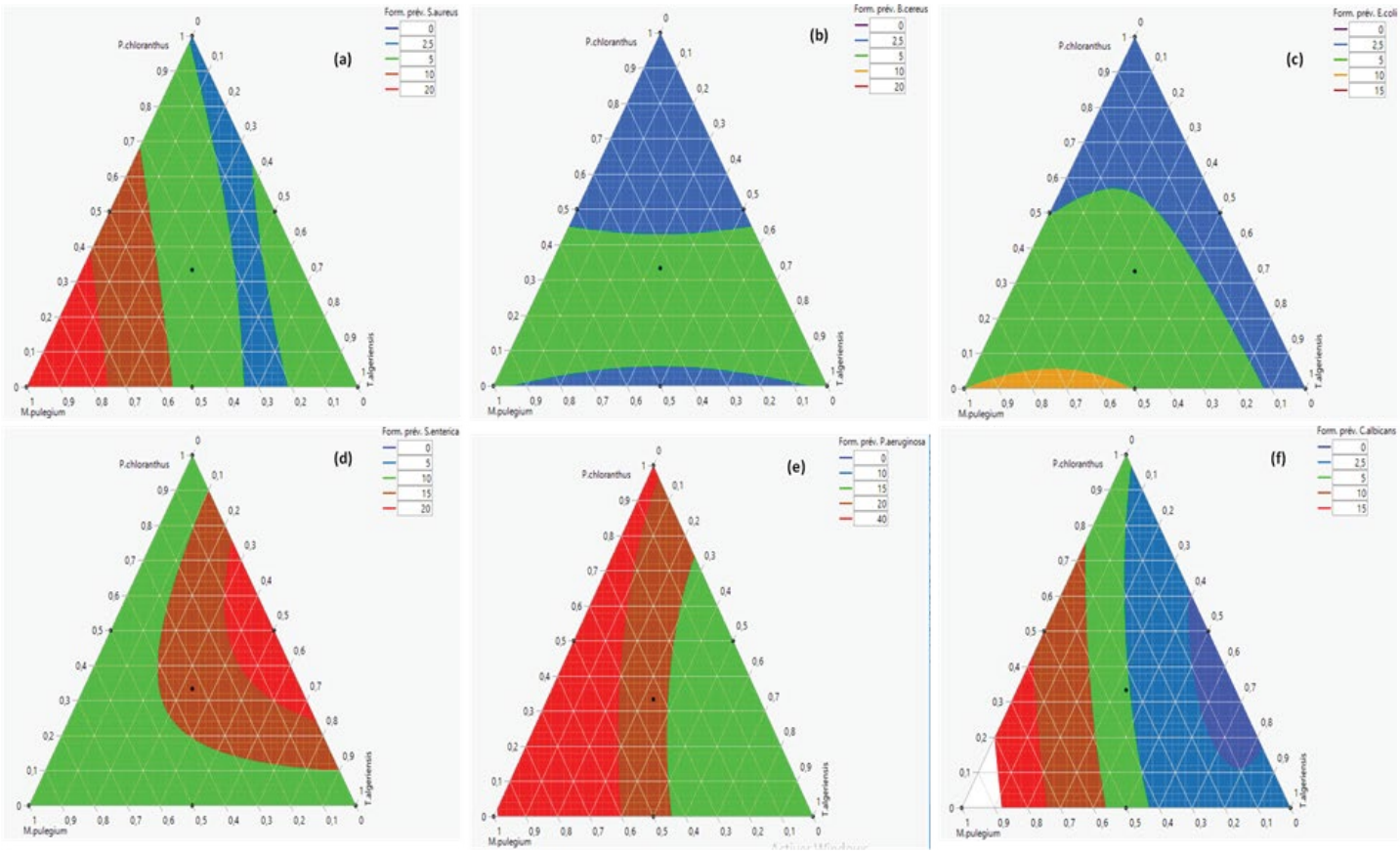


Figure 3: Ternary contour plots of the responses (MICs) for *S. aureus* (**a**), *B. cereus* (**b**), *E. coli* (**c**), *S. enterica* (**d**), *P. aeruginosa* (**e**) *and C. albicans* (**f**).

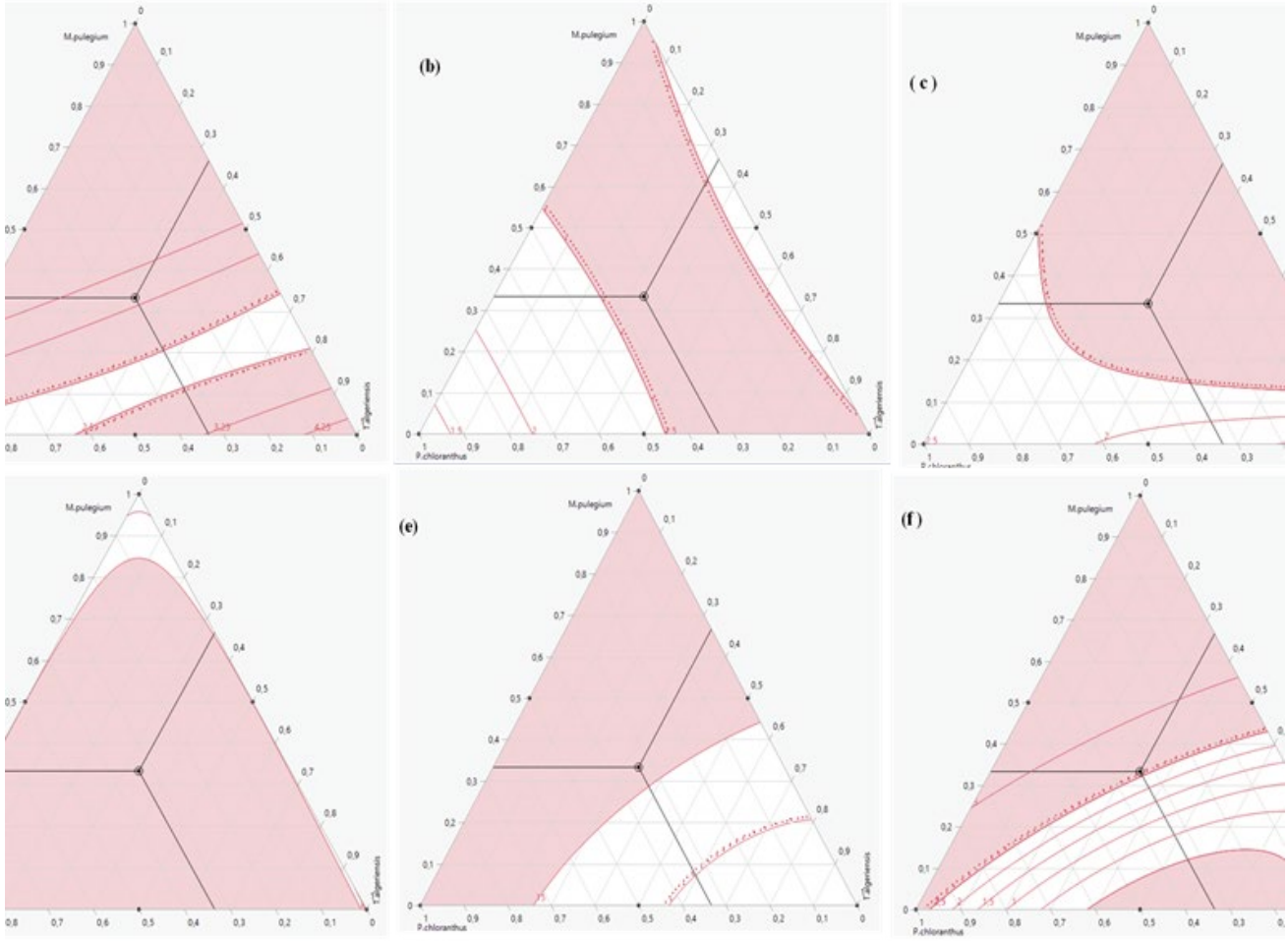


Figure 4: Optimal design regions for the antibacterial effect of EOs mixture against *S. aureus* (*a*), *B. cereus* (b), *E. coli* (c), *S. enterica* (d), *P. aeruginosa* (e) *and C. albicans* (f).

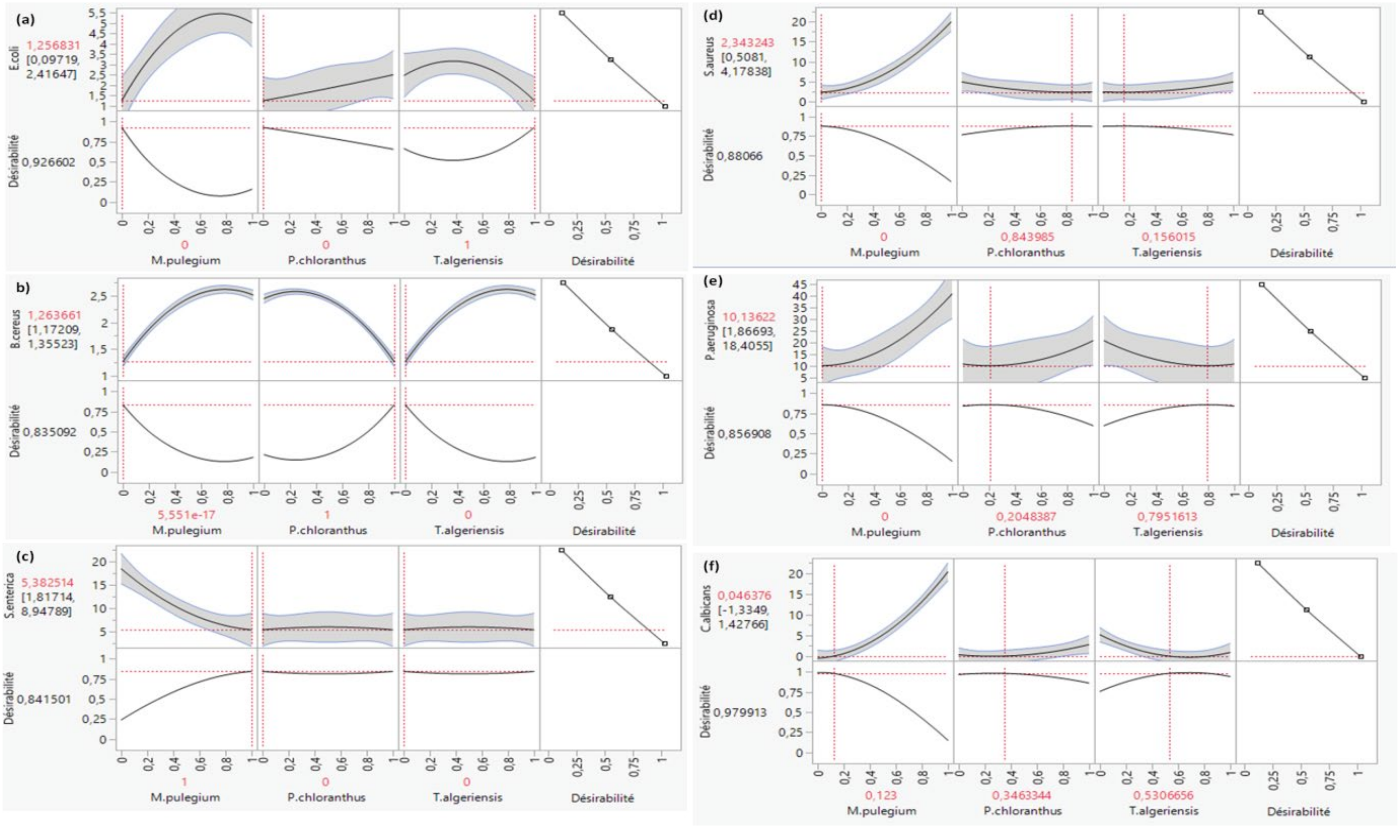


Figure 5: Desirability plot showing the precise proportions of three studied EOs leading to the optimal antibacterial activity against *S. aureus (a)*, *B. cereus* (b), *E. coli* (c), *S. enterica* (d), *P. aeruginosa* (e) *and C. albicans* (f).

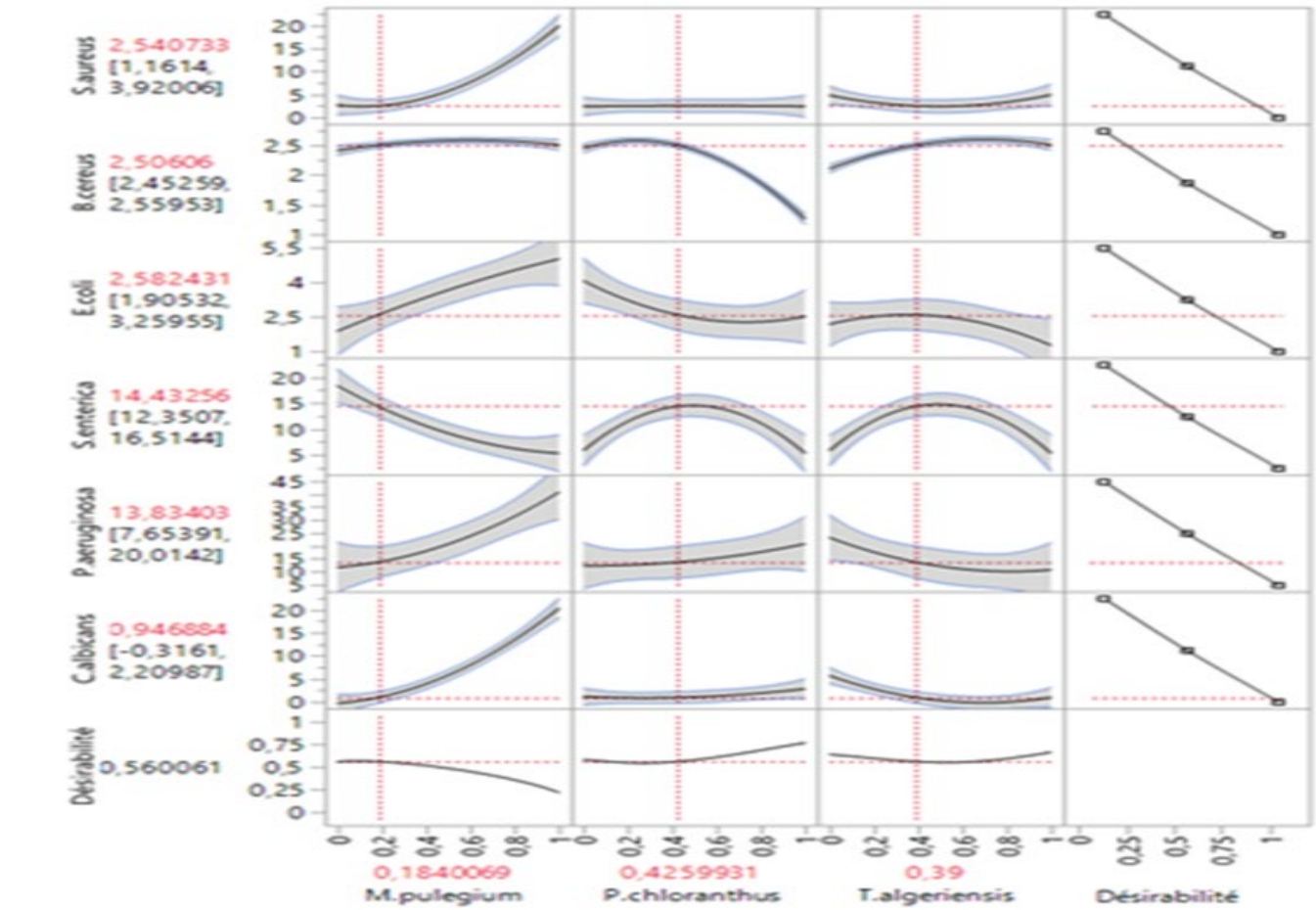
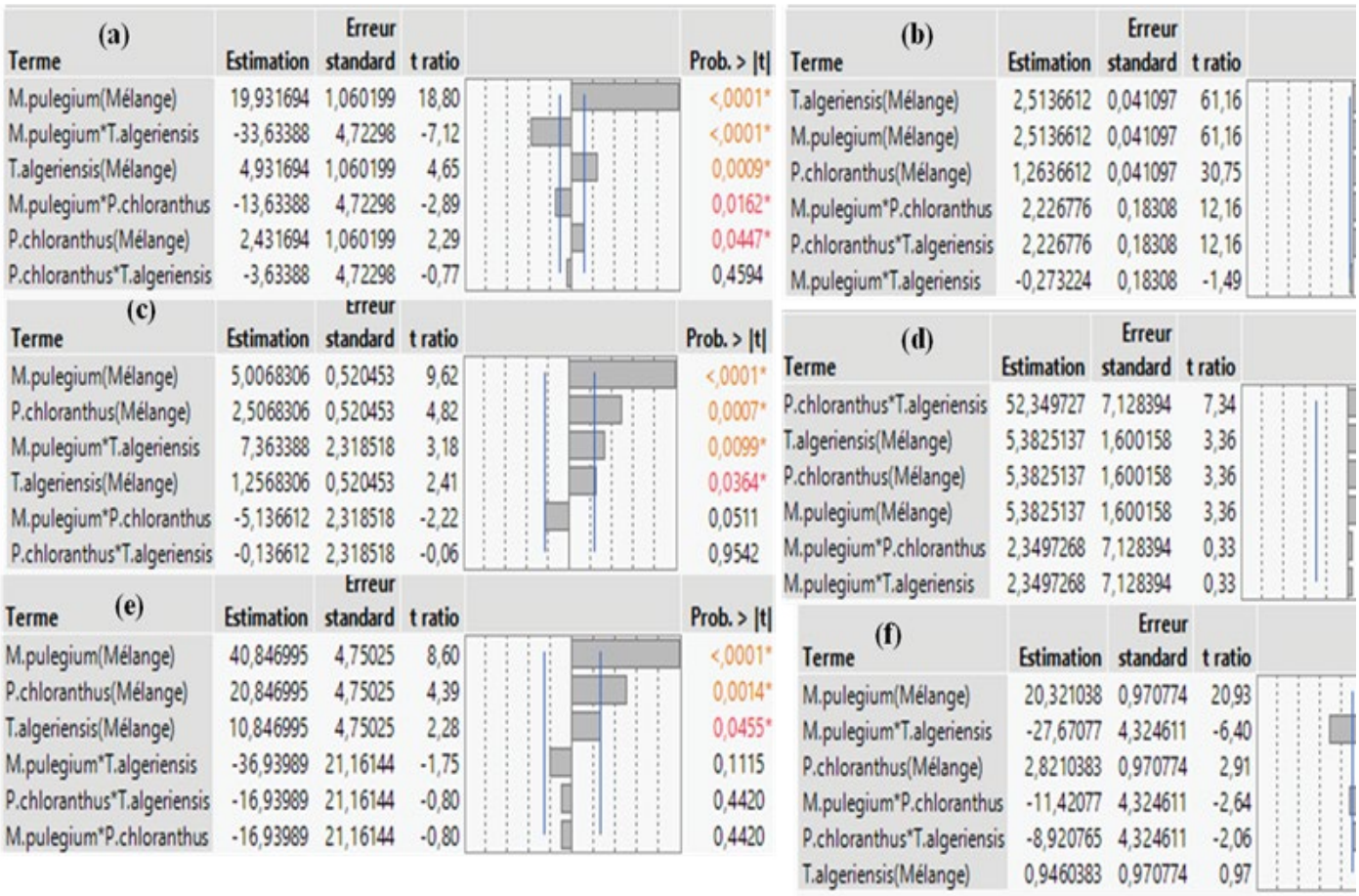


Figure 6: Desirability plot showing the precise proportions of three studied EOs leading to the desired MIC value equal to 2. 5 for the strain *S. aureus (d)*, *B. cereus* (b), *E. coli* (a) ; less then 15 for *S. enterica* (c), *P. aeruginosa* (e) and less then 1 against *C. albicans* (f).



(a)

Terme	Estimation	Erreur standard	t ratio	Prob. > |t|
M.pulegium(Mélange)	19,931694	1,060199	18,80	<,0001*
M.pulegium*T.algeriensis	-33,63388	4,72298	-7,12	<,0001*
T.algeriensis(Mélange)	4,931694	1,060199	4,65	0,0009*
M.pulegium*P.chloranthus	-13,63388	4,72298	-2,89	0,0162*
P.chloranthus(Mélange)	2,431694	1,060199	2,29	0,0447*
P.chloranthus*T.algeriensis	-3,63388	4,72298	-0,77	0,4594

(b)

Terme	Estimation	Erreur standard	t ratio
T.algeriensis(Mélange)	2,5136612	0,041097	61,16
M.pulegium(Mélange)	2,5136612	0,041097	61,16
P.chloranthus(Mélange)	1,2636612	0,041097	30,75
M.pulegium*P.chloranthus	2,226776	0,18308	12,16
P.chloranthus*T.algeriensis	2,226776	0,18308	12,16
M.pulegium*T.algeriensis	-0,273224	0,18308	-1,49

(c)

Terme	Estimation	Erreur standard	t ratio	Prob. > |t|
M.pulegium(Mélange)	5,0068306	0,520453	9,62	<,0001*
P.chloranthus(Mélange)	2,5068306	0,520453	4,82	0,0007*
M.pulegium*T.algeriensis	7,363388	2,318518	3,18	0,0099*
T.algeriensis(Mélange)	1,2568306	0,520453	2,41	0,0364*
M.pulegium*P.chloranthus	-5,136612	2,318518	-2,22	0,0511
P.chloranthus*T.algeriensis	-0,136612	2,318518	-0,06	0,9542

(d)

Terme	Estimation	Erreur standard	t ratio
P.chloranthus*T.algeriensis	52,349727	7,128394	7,34
T.algeriensis(Mélange)	5,3825137	1,600158	3,36
P.chloranthus(Mélange)	5,3825137	1,600158	3,36
M.pulegium(Mélange)	5,3825137	1,600158	3,36
M.pulegium*P.chloranthus	2,3497268	7,128394	0,33
M.pulegium*T.algeriensis	2,3497268	7,128394	0,33

(e)

Terme	Estimation	Erreur standard	t ratio	Prob. > |t|
M.pulegium(Mélange)	40,846995	4,75025	8,60	<,0001*
P.chloranthus(Mélange)	20,846995	4,75025	4,39	0,0014*
T.algeriensis(Mélange)	10,846995	4,75025	2,28	0,0455*
M.pulegium*T.algeriensis	-36,93989	21,16144	-1,75	0,1115
P.chloranthus*T.algeriensis	-16,93989	21,16144	-0,80	0,4420
M.pulegium*P.chloranthus	-16,93989	21,16144	-0,80	0,4420

(f)

Terme	Estimation	Erreur standard	t ratio
M.pulegium(Mélange)	20,321038	0,970774	20,93
M.pulegium*T.algeriensis	-27,67077	4,324611	-6,40
P.chloranthus(Mélange)	2,8210383	0,970774	2,91
M.pulegium*P.chloranthus	-11,42077	4,324611	-2,64
P.chloranthus*T.algeriensis	-8,920765	4,324611	-2,06
T.algeriensis(Mélange)	0,9460383	0,970774	0,97

Figure 7: The effect of different combinations of studied essential oils on MICs values against *S. aureus* **(a)**, *B. cereus* **(b)**, *E. coli* **(c)**, *S. enterica* **(d)**, *P. aeruginosa* **(e)** *and C. albicans* **(f)**.

with a desirability of 100% *T. algeriensis*, 100% *P. chloranthus* and 100% *M. pulegium*, respectively whereas, the precise proportions of binary EOs mixtures leading to the optimal antibacterial activity against *S. aureus* (Figure 5d) and *P. aeruginosa* (Figure 5e) were 16% *T. algeriensis*-84% *P. chloranthus* and 80% *T. algeriensis*-20% *P. chloranthus*, respectively. The optimal anticandidal was obtained by realizing ternary EO mixture consisting 12% *M. pulegium*, 35% *P. chloranthus* and 53% *T. algeriensis* (Figure 5f).

And to finish, Figure 6 shows the plot desirability of the precise proportions of three studied EOs leading to the desired MIC value equal to 2.5 against the strain *S. aureus* (Figure 6d), *B. cereus* (Figure 6b), *E. coli* (Figure 6a); less than 15 for *S. enterica* (Figure 6c), *P. aeruginosa* (Figure 6e) and less than 1 against *C. albicans* (Figure 6f). Thus, the point of the optimal mixture consisting 19% *M. pulegium*, 41% *P. chloranthus* and 40% *T. algeriensis*. These results have suggested a possible synergistic or additive effect between these three Eos. The point on the optimal mixture was used to confirm the validity of the postulated model and there is any significant difference between the predicted and experimental responses. Hence, the optimal mixture should be considered as a potential alternative for control of food safety.

Optimization of the anti-bacterial and anti-candidal effect: In other to predict the antibacterial combined effect of *M. pulegium*, *T. algeriensis* and *P. chloranthus* essential oils and define the optimal mixture, the augmented simplex-centroid mixture design was chosen.

The mixtures design of the three studied EOs and the experimental responses (MICs) obtained on each studied strain are listed in Table 3. Therefore, the JMP software coefficient estimation section shows the values of the model coefficients (Figure 7). The effect is statistically significant, when the p-value was less than 5%. Generally, the effects of the pure components are significant, except *T. algeriensis* EO against *C. albicans*. as shown in Figures 7a-7f with p-value=0.352. Furthermore, negative sign of a coefficient shows the response decrease while the factors values increase while the positive sign indicates that the ability of a factor to increase the response variable. As the aim of this study is to minimize the MIC values, a negative sign of the coefficient is targeted to increase the antibacterial effect. In fact, the interaction between mixture components can produce four types of outcomes: indifferent, additive, antagonistic and synergistic outcomes. Figure 7 showed significant synergistic or additive effects in some binary mixtures, in particular *M. pulegium-T. algeriensis* and *M. pulegium-P. chloranthus* against *S. aureus* (a), and *C. albicans* (f), whereas the binary mixture *T. algeriensis-P. chloranthus* is produced indifferent or antagonistic outcomes. The indifferent effect is against *S. aureus* (a), *E. coli* (c), *P. aeruginosa* (e) and *C. albicans* (f); the antagonistic outcome is against *B. cereus* (b) and *S. enterica* (d).

Besides, Table 4 predicted the special cubic models for the experimental responses (MICs) from EOs mixtures. They describe the relationship between the MICs values and the fraction of each EOs. The prediction purpose for the R^2 values is higher than 0.74 which considered adequate [30]. The relationship between

Table 4: Predicted models of the responses (MICs) obtained for each bacteria.

Bacteria	Predicted models
Staphylococcus aureus	$Y=19.94 X_1+ 2.43 X_2 + 4.9 X_3-13.63 X_1 X_2 - 33.63 X_1 X_3 -3.63 X_2 X_3$
Bacillus cereus	$Y=2.5 X_1+ 1.26 X_2+ 2.5 X_3+ 2.22 X_1 X_2- 0.27 X_1 X_3 + 2.22 X_2 X_3$
Escherichia coli	$Y=5 X_1+ 2.5X_2+ 1.25 X_3-5.13 X_1 X_2+7.37X_1 X_3-0.13X_2 X_3$
Salmonella enterica	$Y= 5.38 X_1+ 5.38 X_2+ 5.38 X_3+ 2.34 X_1 X_2+ 2.34 X_1 X_3 + 52 X_2 X_3$
Pseudomonas aeruginosa	$Y=40.84 X_1+ 20.84 X_2 + 10.84 X_3 - 16.93 X_1 X_2 - 36.93 X_1 X_3 - 16.93 X_2 X_3$
Candida albicans	$Y=20.32 X_1 + 2.82 X_2 + 0.94 X_3 - 11.42 X_1 X_2 - 27.67 X1 X3 - 8.92 X_2 X_3$

the observed and predicted values (Figure 2) predicted a linear curve. These results were confirmed a good agreement between the predicted and the experimental values.

CONCLUSION

In conclusion, these results showed that *T. algeriensis*, *M. pulegium*, *P. chloranthus* EOs alone or combined are effective against the six microbial foodborne strains (*B. cereus*, *S. enterica*, *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*). The screening of antimicrobial activity of the three studied EOs allowed us, firstly, to confirm the highest antibacterial activity of *T. algeriensis* EO followed by *P. chloranthus* EO against *E. coli*, *P. aeruginosa* and *C. albicans* and the highest antibacterial activity of *P. chloranthus* EO followed by *T. algeriensis* EO against *S. aureus* and *B. cereus*; however, results show the lowest antibacterial activity of *M. pulegium* against all studied strains. Secondly, we observed a great potential of *M. pulegium* EO in combined with *T. algeriensis* or *P. chloranthus* against *S. aureus*, and *C. albicans*. This result was explained by a possible synergistic or additive of double and triple combinations of EOs constituents. Especially, the combination between 19% *M. pulegium*, 41% *P. chloranthus* and 40% *T. algeriensis* consisting the optimal ternary mixture Eos. These results indicate the possible use of the essential oils on food system as antimicrobial agents. In this way, these results should be a promising approach and an interesting for the optimization of food preservation, considering both sensory quality of food and economic aspects.

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