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Proceeding Paper

Mixture Design as a Tool for Optimization of Antimicrobial Activity of Selected Essential Oils †

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Abstract: The study aimed to use a statistical method of mixture design to optimize the antimicrobial activity of Tea Tree (*Melaleuca alternifolia*), Rosewood (*Aniba rosaeodora*), and Lavender (*Lavandula hybrida*) essential oils against *Escherichia coli* PCM 2057, *Listeria monocytogenes* PCM 2191, and *Rhodotorula mucilaginosa* EPSC001. The antimicrobial activity of used essential oils and their mixtures were evaluated by the disc diffusion method. Moreover, the antioxidant activity of tested essential oils was determined by the DPPH• and CUPRAC methods, and total phenolic content was measured using the Folin–Ciocalteu method. Tea tree essential oil was characterized by the highest total phenolic content (0.59 ± 0.05 mg GAE/g) followed by lavender oil (0.27 ± 0.05 mg GAE/g), and rosewood oil (0.11 ± 0.02 mg GAE/g). The first two oils also had similar antioxidant activity. Furthermore, essential oil from the tea tree exhibited the highest antimicrobial activity against tested microorganisms, and based on the mixture design approach, the aforementioned volatile oil participated in optimized mixtures in the greatest amount.

Keywords: antimicrobial activity; antioxidant activity; essential oils; mixture design

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1. Introduction

Ensuring food safety is a very important element of food production. In order to maintain the microbiological purity of food products, mainly food additives are used. Unfortunately, some of these substances arouse controversy among consumers. A natural alternative to chemically obtained food additives is the use of essential oils (volatile oils) whose biological activities, including antimicrobial and antioxidant properties, have been confirmed for many of them and make them suitable for food preservation and other applications [1].

The study aimed to use a statistical method of mixture design to optimize the antimicrobial activity of Tea Tree (*Melaleuca alternifolia*), Rosewood (*Aniba rosaeodora*), and Lavender (*Lavandula hybrida*) essential oils against *E. coli* PCM 2057, *L. monocytogenes* PCM 2191, and *R. mucilaginosa* EPSC001.

2. Materials and Methods

2.1. Materials

Tea tree, rosewood, and lavender essential oils (Bianca Cosmetics Lab, Cegłów, Poland) were purchased in a local pharmacy in Warsaw (Poland). All used chemicals were purchased from Sigma-Aldrich (Poznań, Poland) and Avantor Performance Materials Poland S.A. (Gliwice, Poland). Culture media were purchased from BTL Sp. z o. o. (Łódź, Poland).

2.2. Microorganisms

In the study, the following microorganisms were used: *R. mucilaginosa* EPSC001 isolated and identified in the Department of Chemistry (WULS, Poland), *E. coli* PCM 2057, and *L. monocytogenes* PCM 2191 purchased from the Polish Collection of Microorganisms (PCM) of Institute of Immunology and Experimental Therapy Polish Academy of Sciences (Wrocław, Poland).

2.3. Determination of Total Phenolic Content and Antioxidant Activity by the DPPH· and CUPRAC Methods

Firstly, methanolic extracts of essential oils were prepared by *n*-hexane/methanol extraction. Total phenolic contents in the obtained methanolic extracts were determined using the Folin–Ciocalteu method according to Rybak et al. [2]. The content of phenolic compounds was calculated as gallic acid equivalents (mg GAE/g of essential oil).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method was used to determine the antioxidant activities of essential oils and their methanolic extracts [3]. The CUPRAC (cupric ion reducing antioxidant capacity) method was also used to compare the antioxidant activity of methanolic extracts [3]. Antioxidant activity was expressed as the percentage of radical scavenging, and TEAC (Trolox Equivalent Antioxidant Capacity; µmol Trolox/g of essential oil) values were also calculated.

2.4. Mixture Design

The experiment used a simplex-lattice plan augmented with interior points and centroid. The experimental design is shown in Table 1, which was adapted and then analyzed with Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). Mixtures of essential oils were prepared according to Table 1, and their antimicrobial properties against 3 microorganisms were evaluated.

Table 1. Simplex-Lattice Design, experimentally measured inhibition zone diameters, approximated values, and residues for three tested microorganisms.

Exp. No.	Essential Oil			*E. coli* PCM 2057			*L. monocytogenes* PCM 2191			*R. mucilaginosa* EPSC001		
				Inhibition Zone Diameters								
	A	B	C	Measured	Approx.	Residues	Measured	Approx.	Residues	Measured	Approx.	Residues
1	1.00	0.00	0.00	11.33	10.85	0.48	11.33	11.34	−0.01	11.00	11.31	−0.31
2	0.00	1.00	0.00	19.00	19.29	−0.29	18.33	17.98	0.35	17.33	16.82	0.51
3	0.00	0.00	1.00	13.33	11.96	1.37	12.33	12.43	−0.10	11.33	11.31	0.02
4	0.50	0.50	0.00	16.33	15.07	1.26	11.33	10.82	0.51	15.33	15.13	0.20
5	0.50	0.00	0.50	11.33	11.40	−0.07	9.33	9.27	0.06	14.00	14.28	−0.28
6	0.00	0.50	0.50	17.33	15.63	1.70	15.33	14.91	0.42	16.67	16.13	0.54
7	0.67	0.17	0.17	12.33	12.44	−0.11	9.33	9.73	−0.40	11.33	10.64	0.69
8	0.17	0.67	0.17	16.33	16.66	−0.33	12.33	13.82	−1.49	11.33	13.10	−1.77
9	0.17	0.17	0.67	10.67	13.00	−2.33	11.33	11.45	−0.12	10.67	10.98	−0.31
10	0.33	0.33	0.33	12.33	14.03	−1.70	11.67	10.92	0.75	9.33	8.64	0.69

A—Lavender Essential Oil; B—Tea Tree Essential Oil; C—Rosewood Essential Oil.

2.5. Evaluation of Antimicrobial Properties of Essential Oils by the Disc Diffusion Method

Antimicrobial activity of essential oils and their mixtures was evaluated by the disc diffusion method. Mueller–Hinton agar (BTL Sp. z o. o., Łódź, Poland) was used for bacteria, and Sabouraud dextrose agar (4% glucose, 1% peptone, 1.5% agar, pH = 5.6) for yeast. Bacterial or fungal 0.5 McFarland density suspensions were spread over the surface of the agar plates, and 6 mm blank discs soaked with 10 µL of essential oil mixture were placed. Agar plates were incubated for 16–18 h at 37 °C for bacteria or 48 h at 28 °C for fungi. Subsequently, inhibition zone diameters were measured.

2.6. Statistical Analysis

Statistical analyses were performed with Statistica 13.3 software. One-way analysis of variance (ANOVA) and Tukey's test were used to determine significant differences among means ($p < 0.05$).

3. Results and Discussion

In the first stage of the experiment, the total phenolic content, as well as antioxidant properties of three tested essential oils, were assayed. The results are presented in Table 2. Tea tree essential oil was characterized by the highest total phenolic content (0.59 ± 0.05 mg GAE/g), followed by lavender oil (0.27 ± 0.05 mg GAE/g) and rosewood oil (0.11 ± 0.02 mg GAE/g).

In the case of antioxidant activity, increased phenolic content resulted in higher activity, but interestingly, antioxidant properties of tea tree and lavender oils, despite significant differences in TPC values, were comparable, and for the CUPRAC method, it was 6.55 ± 0.78 and 5.99 ± 0.80 µmol Trolox/g EO, for tea tree and lavender oils, respectively.

For the DPPH radical method, properties of tested oils were investigated for both essential oils and their methanolic extracts. According to the adopted measurement methodology, the percentages of antioxidant activities were measured, and they ranged from 7.56% to 12.60%. Similarly to the abovementioned method, rosewood oil had the lowest activity. Trolox equivalent antioxidant capacities were also calculated, and for methanolic extracts, the following values were obtained: 2.01 ± 0.04 (tea tree oil), 1.55 ± 0.16 (rosewood oil), and 2.19 ± 0.15 (lavender oil). Comparable values were obtained for pure essential oils: 2.22 ± 0.23, 1.42 ± 0.40, and 2.30 ± 0.16, respectively.

Other bioactive compounds—terpenoids, can be found in much larger amounts in essential oils. They are especially monoterpenes and sesquiterpenes [1]. Linalool is one of the major compounds in rosewood and lavender oils [4,5], moreover, the latter very often also contains 1,8-cineole [6]. In the case of tea tree oil, γ-terpinene and terpinen-4-ol are found in the largest quantities [7].

Table 2. Total phenolic content and antioxidant activity by means of the DPPH· and CUPRAC methods.

	DPPH				CUPRAC	TPC
	ESSENTIAL OIL		Methanolic Extract			
	AA (%)	TEAC (µmol Trolox/g EO)	AA (%)	TEAC (µmol Trolox/g EO)	TEAC (µmol Trolox/g EO)	mg GA/g EO
Tea Tree	12.14 ± 1.30 ^{A,*}	2.22 ± 0.23 ^{A}	10.93 ± 0.24 ^{A}	2.01 ± 0.04 ^{A}	6.55 ± 0.78 ^{A}	0.59 ± 0.05 ^{A}
Rosewood	7.56 ± 2.29 ^{B}	1.42 ± 0.40 ^{B}	8.29 ± 0.90 ^{B}	1.55 ± 0.16 ^{B}	1.67 ± 0.40 ^{B}	0.11 ± 0.02 ^{C}
Lavender	12.60 ± 0.92 ^{A}	2.30 ± 0.16 ^{A}	11.97 ± 0.85 ^{A}	2.19 ± 0.15 ^{A}	5.99 ± 0.80 ^{A}	0.27 ± 0.05 ^{B}

Abbreviations: AA—antioxidant activity; TEAC—Trolox equivalent antioxidant capacity; EO—essential oil; GA—gallic acid; TPC—total phenolic content. * The values with a different letter (A–C) in a column are significantly different (α = 0.05).

After reviewing the content of phenolic compounds and the antioxidant activity of the essential oils used, the next step was to obtain a mixture of these oils with the greatest ability to inhibit the growth of microorganisms. For this purpose, the augmented simplex-lattice plan was prepared (Table 1), and obtained mixtures were examined as antimicrobial agents against *E. coli* PCM 2057, *L. monocytogenes* PCM 2191, and *R. mucilaginosa EPSC001*. The mean results of inhibition zone diameters were presented in Table 1, as well as approximated, and residual values were calculated. It was shown that tea tree essential oil had the highest antimicrobial activity against tested microorganisms, and the inhibition zone diameters ranged from 17.33 to 19.00 mm. The rest of the evaluated essential oils, namely lavender and rosewood, exhibited moderate activity and had a similar effect on bacteria and yeast. In the case of the former, inhibition zones were approximately 11 mm, and in the latter, they were 11.33–12.33 mm. Based on the performed experiments (Table 1), it was found that high activity was also observed for the mixture of tea tree and rosewood essential oils in a ratio of 1:1. The measured values were statistically analyzed, and ANOVA results for different statistical models (linear, quadratic, special cubic, and cubic) were presented in Tables 3–5.

Table 3. ANOVA results for different statistical models for *E. coli* PCM 2057.

Model	SS	df	MS	F	*p*-Value	R^2	R^2_{adj}
Linear	63.1605	2	31.5802	14.5802	0.0032	0.8064	0.7511
Quadratic	4.7560	3	1.5853	0.6094	0.6434	0.8671	0.7011
Special cubic	6.7985	1	6.7985	5.6540	0.0978	0.9539	0.8618
Cubic	3.2391	2	1.6195	4.3986	0.3195	0.9953	0.9577

SS—sum of square; df—degrees of freedom; MS—mean of square; F—F-values; R^2—coefficient of determination; R^2_{adj}—adjusted coefficient of determination.

Table 4. ANOVA results for different statistical models for *L. monocytogenes* PCM 2191.

Model	SS	df	MS	F	*p*-Value	R^2	R^2_{adj}
Linear	45.4444	2	22.7222	7.5901	0.0177	0.6844	0.5942
Quadratic	17.4328	3	5.8109	6.5982	0.0499	0.9469	0.8806
Special cubic	0.0882	1	0.0882	0.0771	0.7993	0.9483	0.8448
Cubic	1.2727	2	0.6364	0.2944	0.7934	0.9674	0.7070

SS—sum of square; df—degrees of freedom; MS—mean of square; F—F-values; R^2—coefficient of determination; R^2_{adj}—adjusted coefficient of determination.

Table 5. ANOVA results for different statistical models for *R. mucilaginosa* EPSC001.

Model	SS	df	MS	F	*p*-Value	R^2	R^2_{adj}
Linear	24.0494	2	12.0247	1.8474	0.2268	0.3455	0.1585
Quadratic	3.7545	3	1.2515	0.1197	0.9438	0.3994	0.0000
Special cubic	36.8802	1	36.8802	22.4557	0.0178	0.9292	0.7877
Cubic	3.7239	2	1.8620	1.5476	0.4942	0.9827	0.8444

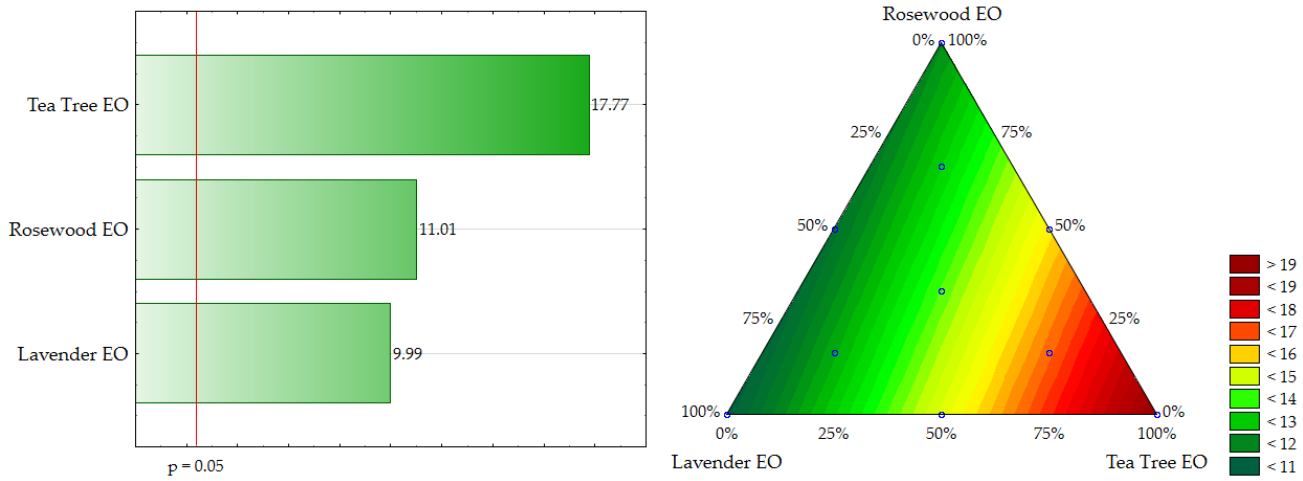
SS—sum of square; df—degrees of freedom; MS—mean of square; F—F-values; R^2—coefficient of determination; R^2_{adj}—adjusted coefficient of determination.

Using the *p*-values, and coefficients of determination (R^2) prepared models were compared. At the level of $p < 0.05$, statistical significance was observed for the linear model for *E. coli*, linear and quadratic models for *L. monocytogenes*, and the special cubic model for *R. mucilaginosa*. In the case of *L. monocytogenes* for further analyses, the quadratic model was chosen due to the higher R^2 value (0.9469) in comparison with the linear model ($R^2 = 0.6844$).

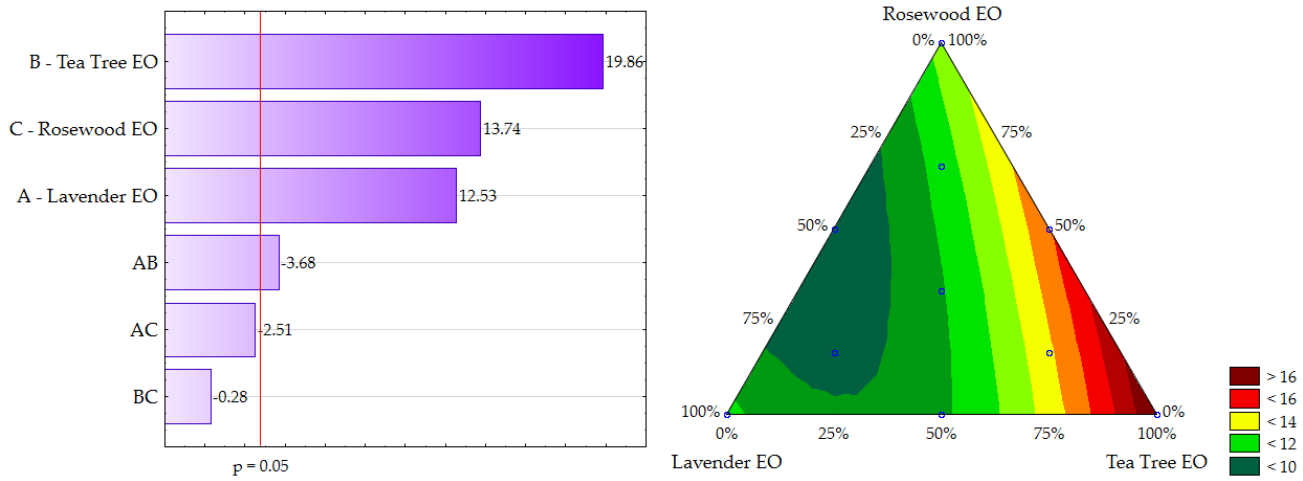
The obtained results fitted to the abovementioned models were presented with the use of Pareto charts and contour plots (Figure 1). When analyzing the results presented in the paper, it can be seen that tea tree oil was the most responsible for microorganisms' growth inhibition. In each of the presented contour plots, the most red/burgundy color was assigned to this oil. This means that in the preparation of the antimicrobial mixture, the tea tree oil should be in the highest content. This is also confirmed by the Pareto charts, which showed that this oil was the most important factor among them and their interactions in the case of non-linear models.

The mixture design approach was applied by many researchers in different disciplines of science and has also been investigated in the design of essential oil blends. Baj et al. [8] optimized the composition of a mixture of *Ocimum basilicum* L., *Origanum majorana* L. and *Rosmarinus officinalis* L. to high antioxidant activity. For the same purpose, a mixture of *Apium graveolens* L., *Thymus vulgaris* L., and *Coriandrum sativum* L. essential oils was optimized using the simplex-lattice mixture design [9].

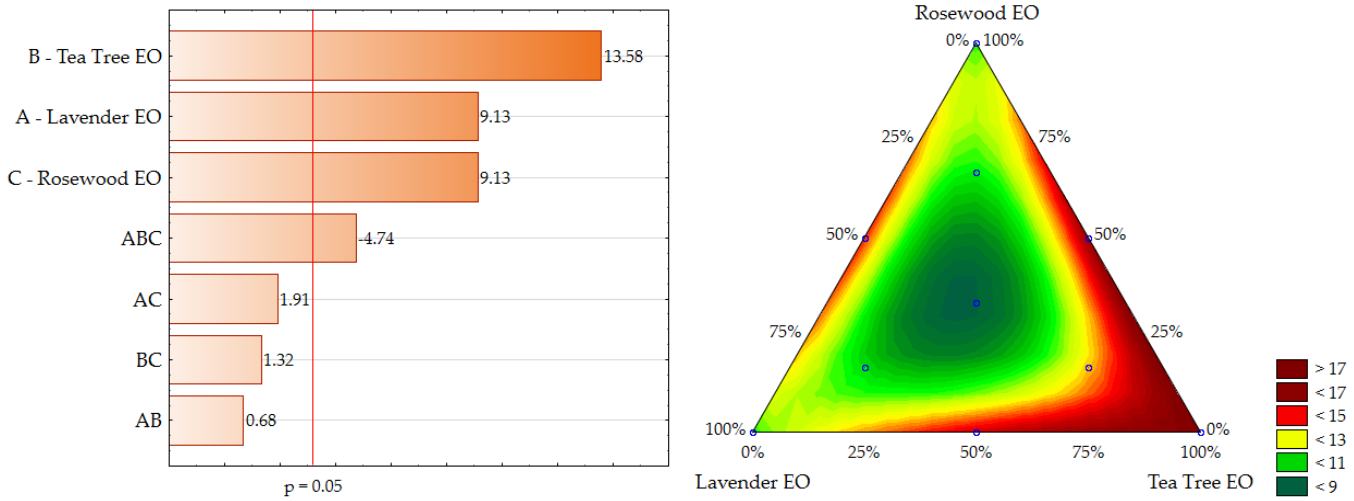
The described statistical method was also suitable in the research of Fadil et al. [10]. The authors compared the mixtures of *T. vulgaris* L., *R. officinalis* L., and *Myrtus communis* L. essential oils in the treatment of *Salmonella typhimurium*, where the interaction between two ingredients was found, and the optimal formulation consisted of 55% of thyme and 45% of myrtle volatile oils, respectively.



(**a**)



(**b**)



(**c**)

Figure 1. Optimization of antimicrobial activity by means of mixture design, presented as Pareto charts and contour plots for a: (**a**) Linear model for *E. coli* PCM 2057, (**b**) quadratic model for *L. monocytogenes* PCM 2191, (**c**) special cubic model for *R. mucilaginosa* EPSC001.

It is well known that essential oils are complex mixtures, hence, the antimicrobial activity cannot be attributed to one particular chemical compound. Phenolic compounds and terpenoids may have a synergistic effect against microorganisms, and the mechanism of their action is linked with cell wall lysis, leakage of intracellular ingredients, and finally, results in cell death [10].

4. Conclusions

These experiments confirmed the possibility of using statistical methods, and in the current study, mixture design with the use of the simplex-lattice plan to develop an optimal essential oils blend with high antimicrobial activity. A natural progression of this work is to analyze the compositions of the essential oils. Further research should also focus on determining possible synergistic effects of tested volatile oils, as well as on establishing the mechanisms of action of compounds included in obtained mixtures on microorganisms.

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