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Statistical methods of data analysis in obtaining thyme oil-loaded nanoemulsions as a potential skin antiseptic

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Abstract
The two aims of this study were to obtain stable thyme-oil loaded nanoemulsions using the statistical design of experiment method (DOE) and to confirm their antimicrobial and disinfecting properties. Thyme oil was used as the oil phase, ECO Tween®80 acted as an emulsifier, and the rest of the formulation was deionized water. Ultrasonication was chosen as the method of obtaining the nanoemulsions. It was checked whether the input parameters (oil concentration, emulsifier concentration, amplitude, and sonication time) had a significant impact on the output parameters (nanoemulsion particle size, polydispersity index, viscosity, and stability over time). For the formulations selected on the basis of the statistical data analysis, the values of minimum inhibitory concentrations (MIC) and minimum biocidal concentrations (MBC/MFC) were determined in relation to 10 bacterial strains and 10 strains of fungi (filamentous fungi, yeast-like fungi). The results obtained from the statistical analysis showed that the optimal concentration of the thyme oil in nanoemulsion should amount up to 2%. Biological studies proved that the obtained formulation had stronger antibacterial and antifungal activity compared to pure oil. Moreover, it was shown that the nanoemulsion caused a reduction of >5 log of bacterial strains (*S. aureus, P. aeruginosa*) and >4 log of fungal strains (*C. albicans*) after 30 minutes, a level required for disinfectants.

Keywords
nanoemulsions, thyme oil, antimicrobial activity, disinfectant, design of experiment

1. INTRODUCTION

Silver nanoparticles have been known and used to control pathogenic microorganisms for a long time (Bruna et al., 2021; Rezvani et al., 2019). They also have anti-inflammatory and antioxidant properties, as demonstrated in the papers by some authors (Bold et al., 2022). Owing to their special properties, silver nanoparticles are used to manufacture medical supplies (dressings, antiseptics for wounds and mucous membranes), materials intended for contact with food, toothpastes, cosmetics, textiles (sports clothing, socks), shoes, plastics, as well as building materials (paints) (Świdwińska-Gajewska and Czerczak, 2014).

The ever-growing use of nano silver in consumer products, water disinfection, and healthcare facilities raises concerns about the public and environmental safety of this nanomaterial. The current omnipresence of nano silver can result in repeated exposure via different routes (skin, inhalation, ingestion), which can lead to serious health effects.

The available literature data shows that silver nanoparticles may have toxic and genotoxic effects on mammalian cells (D'arcy, 2019; Jia et al., 2020; Zhang et al., 2016). There also are alarming reports on the harmful effect of silver nanoparticles on the reproduction of experimental animals (Castellini et al., 2014; Tabandeh et al., 2022). Moreover, it has also been demonstrated that exposure to silver nanoparticles can be neurotoxic and affect cognitive functions by causing short-term memory and working memory disorders (Liu et al., 2012; Strużyńska and Skalska, 2018).

Due to the growing problem related to the use of nano silver, it is justified to search for new active substances and preparations containing them, which can be used to control pathogenic microorganisms and which have other biological properties similar to nano silver. The substances of plant origin, including essential oils, seem to be such substances.

As shown in literature data and own papers (Fani and Kohanteb, 2017; Mahboubi et al., 2017; Marchese et al., 2016; Miastkowska et al., 2020; Michalczyk and Ostrowska, 2021; Šegvić Klarić et al., 2007), *Thymus vulgaris* essential oil is one of the oils having the strongest effect and the widest spectrum of activity against pathogenic microorganisms.

Thymus vulgaris is a flowering plant of the *Lamiaceae* family and it originates in Southern Europe. It is an evergreen shrub with small, strongly aromatic, grey-green leaves and with purple or pink bunches of (Hosseinzadeh et al., 2015). Thyme has various pharmacological properties, including antimicrobial, anti-inflammatory, analgesic, antispasmodic, anti-

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tussive, carminative, antihypertensive, antidiabetic, and anthelmintic properties (Salehi et al., 2018). It is often used in cooking as a seasoning (Kuete, 2015). Burt et al. (2005) reported that thyme essential oil mainly contains carvacrol, thymol, p-cymene and γ-terpinene. The main components in *Thymus* essential oils are classified as Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (FDA).

Due to the antimicrobial properties of the main constituents, *T. vulgaris* essential oil is effective not only against bacteria and yeasts but also inhibits activity of microscopic filamentous (Fani and Kohanteb, 2017; Mahboubi et al., 2017; Marchese et al., 2016; Miastkowska et al., 2020; Michalczyk and Ostrowska, 2021; Šegvić Klarić et al., 2007). Moreover, it was found that the main components of thyme oil (carvacrol, thymol, p-cymene, and γ-terpinene) were also the sources of its high antioxidant activity (Yildiz et al., 2020). In turn, (Braga et al., 2006) demonstrated that thymol had a significant effect on the control of inflammatory mechanism occurring in numerous infections, which may be important in the proper treatment of wounds.

Considering the anti-inflammatory, antioxidant, and antimicrobial properties of the main constituents of thyme oil, it can be assumed that the oil itself can be a promising active ingredient of preparations with antiseptic and anti-inflammatory properties.

However, it should be emphasized that, apart from the active substance itself, the effectiveness of a given preparation also depends on the form of the carrier (Łętocha et al., 2022; Miastkowska et al., 2020). Nanoemulsions are among the most important carriers of active substances. The advantages of those lipid-based nanosystems include reduction of side effects of essentials oils and improvement of their biological activity. Due to small droplet size and increased surface area, the nanometric formulations influence the transport of the essential oils to the microbial cell membrane. Because of these advantages, they can act as 100% ecological and biodegradable formulations, which additionally are safe for the consumer and the environment (Osman Mohamed Ali et al., 2017; Wang et al., 2007). Nanoemulsions based on thyme oil can become a green alternative to commercial products applied to the skin and containing nano silver with antimicrobial properties (Miastkowska et al., 2020; Michalczyk and Ostrowska, 2021).

The aims of the research were to obtain stable nanoemulsions based on thyme oil, stabilized with Eco-Polysorbate 80, and to confirm their antimicrobial and disinfecting properties. The method of statistical experiment planning (DOE) was used to develop the composition of the optimal formulation. Thyme oil itself was also tested for antimicrobial properties. For comparative purposes a commercial preparation containing silver nanoparticles was also analyzed.

2. EXPERIMENTAL

2.1. Chemicals

Thyme oil from *Thymus vulgaris* was purchased from Pollena Aroma (Poland). Sabouraud Dextrose Agar, Sabouraud Dextrose Broth, Mueller-Hinton Broth (MH), Tryptone Soya Agar (TSA) (Oxoid, UK) were used. PBS buffer, L-Lecithin 80, Polisorbate 80, L-Cysteine were purchased from Sigma-Aldrich, USA. Eco-Tween 80 was kindly supplied by Croda (Poland). For comparative purposes, a commercial product containing silver nanoparticles was used and it was obtained from a local pharmacy. Deionized water was used as the solvent.

2.2. Microorganisms

The reference microorganisms used were the following strains of bacteria: *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* NCTC 6749, *Moraxella catarrhalis* ATCC 25238, *Staphylococcus aureus* NCTC 4163, *Staphylococcus epidermidis* ATCC 49134, *Proteus vulgaris* NCTC 4635, *Enterococcus faecalis* ATCC 33186, *Micrococcus luteus* NCTC 7743, *Escherichia coli* ATCC 25922, *Serratia marcescens* ATCC 810; yeast-like fungi: *Candida albicans* ATCC 10231, *Candida glabrata* PCM 2703, *Candida tropicalis* PCM 2709, *Candida lipolytica* PCM 2680, mold fungi: *Aspergillus brasiliensis* ATCC 16404, *Scopulariopsis brevicaulis* DSM 63032, and dermatotrophic fungi: *Trichophyton mentagrophytes* ATCC 9533, *Microsporum gypseum* ATCC 6231, *Microsporum cookei* ATCC 13275, *Trichophyton violaceum* ATCC 11902. The strains were obtained from the following four collections: the American Type Culture Collection (ATCC), the National Collection of Type Cultures (NCTC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and from the Polish Collection of Microorganisms (PCM). The above-mentioned microorganisms are mostly microorganisms that cause skin diseases.

The test fungus strains were stored in the form of slants on Sabouraud Dextrose Agar and the bacteria were stored on Tryptone Soya Agar at 4 °C, then transferred to a fresh medium every 4–8 weeks.

2.3. GC-MS Analysis of thyme oil

The GC/MS chromatographic analysis was performed using a Varian gas chromatograph type 450-GC coupled with a mass detector Varian 320-MS. The equipment was made available by the Central Laboratory of Agroecology, the University of Life Sciences in Lublin. The instruments were connected with a transfer line: a 30 × 0.25 mm quartz column. A capillary column VF-5 ms (a DB-5 equivalent) was used in the study. The dosing volume amounted to 1 µL with the flux split in a 1:100 ratio. Helium in constant flow of 0.5 mL/min was used as the carrier gas. The temperature of

the dispenser was 250 °C. A temperature gradient was applied (50 °C for 1 min, then increased by 4 °C to 250 °C, 250 °C for 10 min). Mass spectra were measured with an electron ionization (EI) mass spectrometer. The electron energy was 70 eV. The temperature of the ion source amounted to –200 °C, and the transfer line to 270 °C. The ions of test compounds were detected in the range from 40 to 400 m/z (a.m.u.) at the scanning speed of 0.8 s/scan. The delay time of ion source switching-on was 180 s. The qualitative analysis was performed on the basis of MS spectra by comparing them with the spectra of the NIST library, Robert Adams library (compounds typical for essential oils), and with LIBR (TR) terpene library (Adams, 2007; Massada, 1976). In the analysis, the non-isothermal Kovats retention index was used and it was based on a series of alkanes (C10-C40).

2.4. Preparation of the nanoemulsions

The nanoemulsions used in this study consisted of thyme oil, deionized water, and Eco-Tween 80. It is a 100% ecological surfactant, belonging to the group of non-ionic surfactants and consisting of ethoxylated sorbitan ester of oleic acid. All samples were prepared by ultrasonication (high energy emulsification). The first step was to prepare a pre-emulsion by combining the water phase with a mixture of thyme oil and a surfactant at a temperature $T \leq 40$ °C with magnetic stirring at a speed of $v = 500$ rpm (IKA C-MAG HS 7). The emulsion was then treated with a probe-type ultrasonic sonicator (UP200 Ht, Hielscher).

2.5. Optimization of composition and process parameters

The composition of the nanoemulsions were optimized with mathematical methods of experiment planning (Statistica®ver. 13, StatSoft, Poland). In order to develop the base nanoemulsion recipe using the statistical method of experimental design (DOE), the central-composition design $3^{(K-p)}$ was used, where p always takes the value 1 and K is the number of variables. In all cases, the statistical significance level was assumed to be $p < 0.05$. The group of input (independent variables) parameters included: essential oil and emulsifier concentration, amplitude and sonification time, whereas the output (dependent variables) were: particle size, polydispersity index, viscosity (mPas·s), and stability. The ranges of the variability of the process input variables are listed in Table 1.

2.6. Physicochemical properties of the obtained formulations

The mean droplet diameter (Z-Ave d.nm) and polydispersity index (PDI) of the resulting formulations were measured using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) at 25 °C. For this purpose, the samples were diluted 1:10 (v/v) with deionized water to avoid multiple scattering effects. The scattering angle was 173 °. Each sample was analyzed in triplicate ($n = 3$) to determine the mean droplet size and standard deviation values. The rheology was measured with a rotational rheometer (Brookfield R/S-CPS Plus Rheometer, UK) at room temperature (25 °C) with a shear rate of up to 500 s^{-1}. The measurement was repeated three times for each sample. Initially, the stability of the nanoemulsion was assessed using the centrifuge method (centrifuge EBA 20 by Hettich Zentrifugen). The samples were centrifuged at 3500 rpm for 10 min. The preparations were then subjected to visual evaluation of the homogeneity of the samples. In the next stage, the formulations were subjected to accelerated aging tests using the variable temperature test (3 cycles, alternately at 40° and 5°).

Table 1. The variability ranges of independent parameters.

Independent variable	The ranges of variability
Essential oil concentration [%]	1, 2, 3
Emulsifier concentration [%]	1, 2, 3
Amplitude [%]	69, 79, 89
Sonication time [min]	1, 2, 3

2.7. Assay of antimicrobial activity

2.7.1. Determination of the MIC and MBC/MFC values for bacteria and yeast-like fungi

The test was performed by serial dilutions in a liquid medium (Michalczyk and Ostrowska, 2021; Wawrzykiewicz et al., 2000). The aim of the study was to determine the minimum concentrations of the tested substances (thyme oil, formulation based on the oil) at which the growth of yeast-like fungi and bacteria in the medium (MIC) was inhibited or the microorganisms (MBC/MFC) were killed. A series of dilutions for each tested substance was prepared in Mueller-Hinton Broth (bacteria) or Sabouraud Dextrose Broth (fungi) media. The oil and the nanoform based on it were tested in the range of concentrations amounting to 10–0.078 μg/μL. In the study, the essential oil was used with the initial concentration amounting to 100 μg/μL. The nanoform based on the oil had the initial oil concentration amounting to 20 μg/μL. Bacteria were cultured in Mueller Hinton Broth for 24 h at 37 °C and fungi in Sabouraud Dextrose Agar for 48 h at 28 °C. A suspension of the microorganisms at a concentration of 10^6 CFU/mL (CFU = colony forming units) was prepared from each culture and each

dilution of the compound was inoculated in a 1:1 ratio. The growth of the microorganisms (or its lack) was determined visually after incubation for 24 h at 37 °C (bacteria) or for 48 h at 28 °C (fungi). The lowest concentration at which there was no visible growth was taken as minimal inhibitory concentration (MIC). In order to determine the minimum bactericidal or fungicidal concentration (MBC or MFC), one loopful taken from each tube was cultured on an agar medium with inactivates (0.3% lecithin L, 3% polysorbate 80, and 0.1% cysteine L) and incubated for 48 h at 37 °C (bacteria) or at 28 °C (fungi). The lowest concentration at which there was no visible colony growth was taken as MBC or MFC. The experiments on fungi were performed in triplicate and the results obtained were used to calculate the average values of the of the MIC and MBC/MFC.

2.7.2. Determination of the MIC and MFC values for dermatotrophic and mold fungi

The study covered the determination of MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) values for thyme oil and nanoemulsion based on the oil. The tests were performed in the range of concentrations amounting to 0.078–10 μg/μL in line with the cylinder dilution method on agar medium (Michalczyk and Ostrowska, 2021; Wawrzykiewicz et al., 2000). The inoculum in the study was cut with a sterile cork borer (5 mm in diameter), cylinders of agar medium coated with a 10–14-day old homogeneous fungal microculture grown at 25 °C (dermatophytes) and 28 °C (molds). The fungal material was collected from spots equidistant from the colony center to make sure that the obtained inocula contain similar fungal elements in qualitative and quantitative terms. Three holes (5 mm in diameter) were cut out with a pre-sterilized cork borer in the solid medium containing Sabouraud Dextrose Agar. Relevant concentrations of the test substance and the fungal inoculum were incorporated into the holes. Accordingly, the plates with Sabouraud Dextrose Agar without additives were the control. The results were read after 5–7 days of incubation at 25 °C (dermatophytes) or 28 °C (molds) in a thermostat. The concentration of the test substance limiting the growth of fungal colonies to 7 mm, i.e. 2 mm beyond the diameter of the incorporated inoculum, was considered to be the MIC value. The samples in which no fungal growth was observed were considered to be negative. The fungal inocula from those samples were incorporated, similarly to the determination of the MIC, on the plates containing Sabouraud Dextrose Agar medium without the additives. Incubation was carried out for consecutive 7 days at a temperature which was adequate for each strain. The concentration causing complete lack of fungal growth (macroscopic assessment) was considered to be the MFC value. The experiments on fungi were performed in triplicate and the results obtained were used to calculate the average value of MIC and MFC.

2.7.3. Evaluation of the antiseptic effect of thyme oil, nanoemulsion obtained from the oil, and a commercial preparation

In order to assess the antiseptic properties of 1% and 2% thyme oil, the nanoemulsion containing corresponding oil concentrations, and of the commercial preparation, the study was carried out in line with the methodology proposed by El-Sayed and El-Sayed (2021) with slight modifications. The tests were carried out using the following strains: *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231. The study used 24 h cultures of bacterial strains on Tryptone Soya Agar incubated at 36 °C and 48 h cultures of yeast-like fungi on Sabouraud Dextrose Agar with gentamicin and chloramphenicol incubated at 25 °C. Before the proper experiment, the reference suspensions of the strains used in the tests, prepared in a sterile diluent (PBS), were standardized. The optical density of the suspensions was assessed with a densitometer (Biosan, Poland). The optical density of the prepared cell suspensions of bacterial strains was 0.5 according to the McFarland scale. The number of cells in the suspensions was assessed by ten-fold dilution and deep inoculation. The standardized bacterial suspensions contained between 10^8 and 10^{11} CFU/mL. Moreover, *C. albicans* ATTC 10231 yeast strain cell suspension with the optical density equal to the McFarland scale value of 3.0 was prepared. The number of cells in the suspensions was assessed by ten-fold dilution and deep inoculation. The standardized fungi suspensions contained 10^7 CFU/mL. The suspensions of microorganisms obtained as described above were transferred to 9 mL of the tested substances, i.e. thyme oil with the following concentrations: 10 and 20 μg/μL (the oil dissolved in 5 μg/μL Eco-Tween 80 solution), 10 and 20 μg/μL nanoemulsion based on thyme oil, and 100% commercial preparation in the amount of 1 mL, for 15 and 30 minutes. The last step consisted in the preparation of a series of decimal dilutions in PBS diluent and in inoculation of 1 mL portions by deep inoculation on a solid TSA medium (bacteria) or Sabouraud medium (yeast-like-fungi). Grown colonies were counted after 72 hours of incubation at 30 °C. The procedure was the same for each strain and each tested substance. The log reduction was also calculated for the number of bacterial and fungal cells able to grow after the application of the antiseptic against the number of cells not exposed to the antiseptic. The media with microorganisms were incubated under aerobic conditions for 48 hours at 35 °C (bacteria) and 72 hours at 25 °C (yeast-like fungi). Next, the grown colonies were counted and the number of cells in 1 mL of inoculated suspension (CFU/mL) was calculated. The procedure was the same for each strain and each tested substance. The log reduction in the number of viable bacterial cells and fungi after disinfection against the number of cells not exposed to the potential disinfectant was also calculated (Log No–Log Nt; where Log No – initial number of cells in the suspension of test microorganisms, Log Nt – the number

of viable cells of bacteria or fungi obtained after a certain time of contact with disinfectant). The tests were repeated three times and the results obtained were used to calculate the average of log reduction.

3. RESULTS

3.1. Chemical composition

In order to correlate the antimicrobial activity with the chemical composition of the test oil, GC-MS analysis was used. The list of ingredients identified in the oil during GC-MS analyses is presented in Table 2. Thyme oil is characterized by a high content of phenolic compounds (48.48%) and monoterpene (50.37%). Other ingredients account for only 1.15% of its composition. The main ingredients of thyme oil are as follows: thymol (35.21%), p-cymene (20.95%), carvacrol (13.27%), linalool (6.39%), γ-terpinene (4.60%), α-pinene (3.78%), and myrcene (2.70%).

Table 2. Percentage content of individual ingredients in *Thymus vulgaris* oil determined by GC-MS.

Compounds	RT/min	Percentage composition of essential oil
$\alpha-$Thujene	8.120	0.26
$\alpha-$Pinene	8.376	3.78
Camphene	8.946	0.28
$\beta-$Pinene	9.878	0.51
Mentha-2.8-diene	10.046	0.18
Myrcene	10.194	2.70
$\alpha-$Terpinene	11.211	1.44
p-Cymene	11.546	20.95
Limonene	11.663	1.18
1.8-Cineole	11.806	1.50
$\gamma-$Terpinene	12.697	4.60
Linalool oxide	13.218	0.11
Fenchone	13.931	0.11
Linalool	14.225	6.39
Borneol	16.989	3.10
Terpinen-4-ol	17.261	0.14
$\alpha-$Terpineol	17.851	0.11
Linalyl acetate	19.511	0.17
Bornyl acetate	20.875	0.37
Thymol	21.208	35. 21
Carvacrol	21.208	13.27
Caryophyllene	25.265	1.64
Caryophyllene oxide	30.338	0.85
Other		1.15

3.2. Results of statistical analysis

Table 3 shows the specific values of process parameters and the results of physicochemical analyses of each obtained sample.

The results presented in Table 3 were subjected to statistical analysis. The statistical analysis was performed on the basis of one-way analysis of variance (ANOVA). It was checked whether the input parameters significantly affected the output parameters of the thyme oil nanoemulsion. The red line indicates statistically significant parameters at a fixed $p < 0.05$. Figure 1 presents Pareto charts for the applied independent parameters. On their basis, it was concluded that neither the PDI nor the stability depended on any of the proposed input parameters. On the other hand, the parameters that have a statistically significant impact on the size of the obtained particles are the concentration of the thyme oil used and the time of the ultrasonication process, as the dimensions of the linear function. In the case of the influence on viscosity, the group of statistically significant parameters includes the concentration of oil and emulsifier as a linear function and time as a square function dimension. The presence of the quadratic function indicates a strong influence of ultrasonication time on viscosity.

The next point of the statistical analysis was the preparation of approximation profiles (Fig. 2) and saddle charts (Fig. 3), indicating which values of the independent parameters allow to achieve the most desirable values of the dependent variables. The aim of the study was to develop a nanoemulsion recipe characterized by the smallest particle size and viscosity in line with literature data, which clearly indicates that these properties translate into the effectiveness of the formulation (Miastkowska et al., 2020).

The analysis of the approximation profiles (Fig. 2) shows that nanoemulsions with the smallest particle size (approx. 149 nm) and the smallest viscosity (approx. 215 mPas·s) were obtained for an intermediate oil concentration of 2%, the emulsifier concentration of 2.5%, the amplitude value (89%) and the ultrasonication time = 1 min. The graphs also show that the viscosity of the nanoemulsion decreased with increasing oil concentration, while the particle size increased. When the concentration of the emulsifier increased to 2,5%, both the size and viscosity value decreased. In addition, an increase in droplet size was observed with increasing ultrasonication time.

The data presented in Figure 3 shows that in order to obtain a nanoemulsion with the desired physicochemical parameters, such as the smallest droplet size of the internal phase and the lowest viscosity, the concentration of the oil

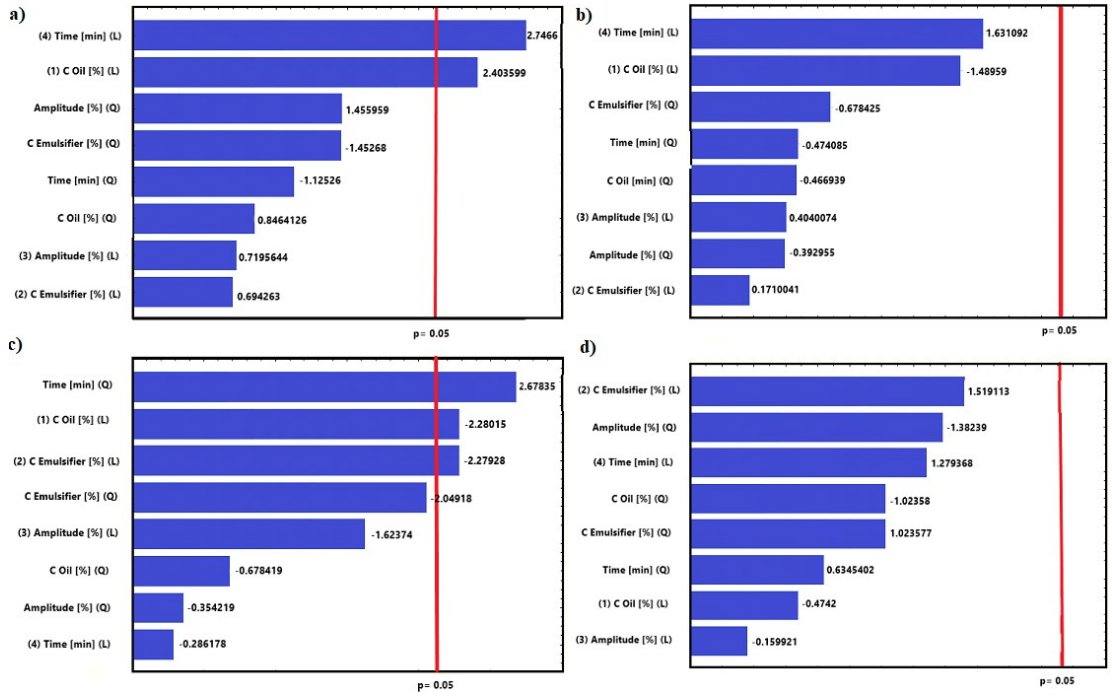

Figure 1. Pareto charts for the influence of input parameters on: a) average droplet size of nanoemulsions; b) polydispersity index (PDI); c) viscosity; d) stability.

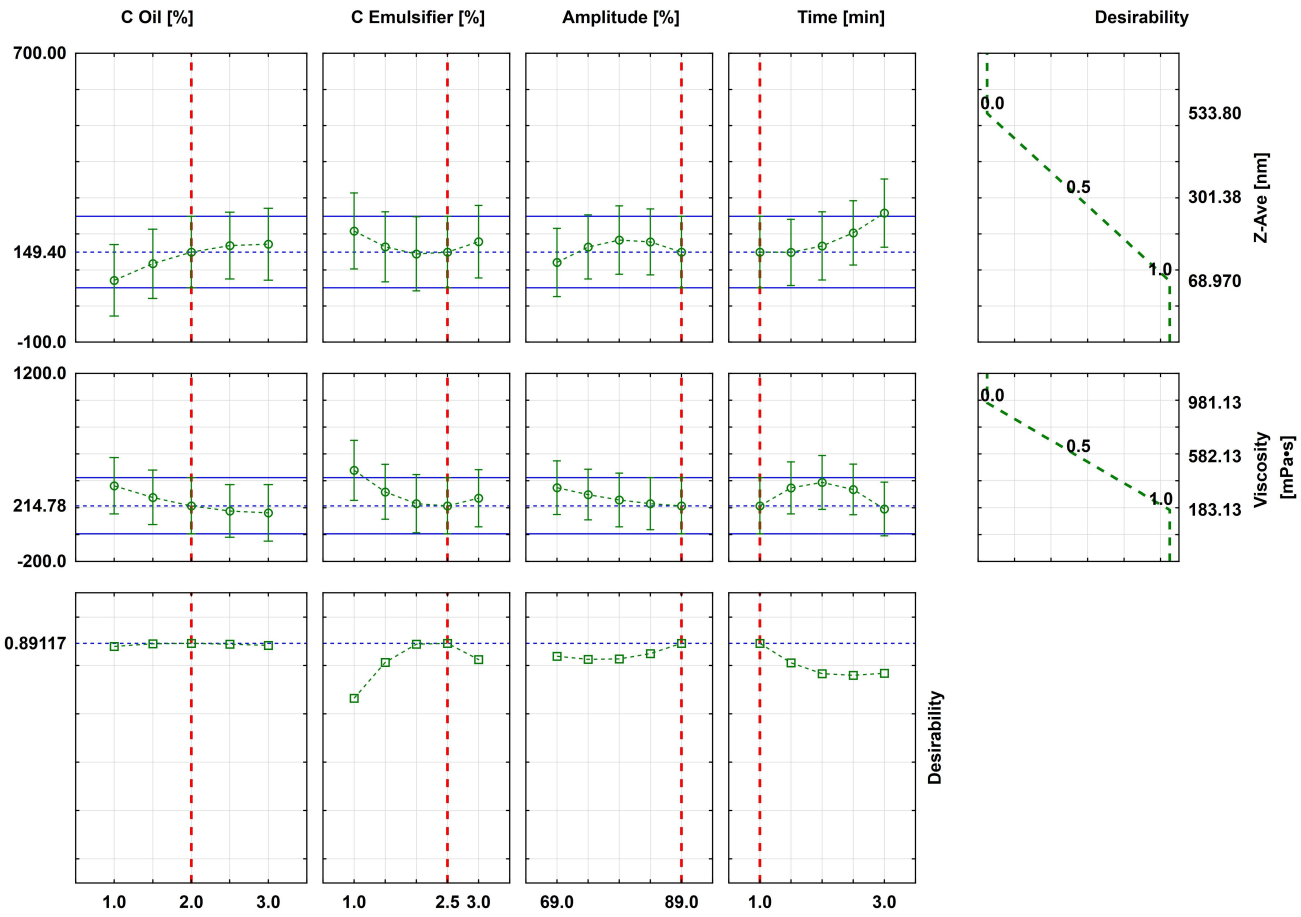

Figure 2. Approximation profiles for the influence of input parameters on: an average droplet size of nanoemulsions (nm) and viscosity (mPas·s).

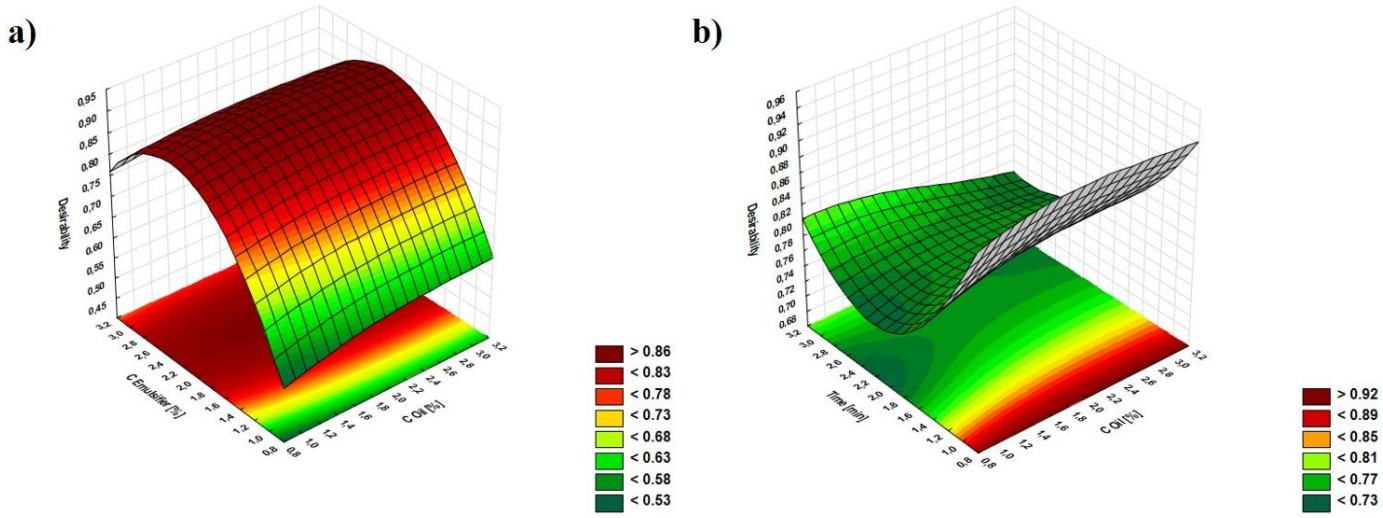

Figure 3. Saddle plots for desirability for: a) emulsifier and oil concentration and b) oil concentration and sonication time.

Table 3. Matrix of the experimental design and experimental data obtained for the dependent variables.

Sample	C Oil [%]	C Emulsifier [%]	Amplitude [%]	Time [min]	Z-Ave [nm]	PDI	Viscosity [mPa·s]	Stability
17	3	3	79	3	533 ± 5	0.137	343 ± 20	1
13	3	2	69	2	185 ± 4	0.182	267 ± 13	1
18	3	3	89	2	212 ± 6	0.076	207 ± 27	1
23	2	2	79	3	324 ± 5	0.385	245 ± 13	1
6	1	2	89	1	196 ± 5	0.089	299 ± 23	1
16	3	3	69	1	96 ± 2	0.082	410 ± 26	1
20	2	1	79	1	223 ± 7	0.219	315 ± 50	0
21	2	1	89	3	300 ± 9	0.39	588 ± 30	1
4	1	2	69	3	137 ± 1	0.085	483 ± 32	1
14	3	2	79	1	145 ± 4	0.182	421 ± 19	1
26	2	3	79	2	183 ± 1	0.031	733 ± 18	1
22	2	2	69	1	84 ± 2	0.156	519 ± 35	1
8	1	3	79	1	177 ± 9	0.249	295 ± 28	1
9	1	3	89	3	127 ± 15	0.569	395 ± 37	1
25	2	3	69	3	263 ± 6	0.141	239 ± 25	1
10	3	1	69	3	–	–	–	0
11	3	1	79	2	–	–	–	0
5	1	2	79	2	98 ± 2	0.200	523 ± 14	1
12	3	1	89	1	–	–	–	0
7	1	3	69	2	68 ± 9	0.530	881 ± 37	1
2	1	1	79	3	180 ± 7	0.283	532 ± 20	1
15	3	2	89	3	233 ± 5	0.235	183 ± 13	1
24	2	2	89	2	165 ± 55	0.144	389 ± 30	1
3	1	1	89	2	170 ± 7	0.202	981 ± 13	1
27	2	3	89	1	162 ± 0.8	0.065	207 ± 17	1

and emulsifier should be in the range of 1.6 to 2.4% and 1.5 to 2.5%, respectively. However, in the case of the influence of the oil concentration and ultrasonication time on desirability, the optimal process time is in the range of 0.8 to 1 min for the entire range of the essential oil concentration.

Based on the design of experiments (DOE) results, samples of nanoemulsions containing thyme oil were prepared (Table 4). The samples were prepared using the maximum amplitude value (89%) and minimum ultrasonication time ($t = 1$ min). As the most optimal concentration of the oil, 2% was chosen and the concentration of the emulsifier at the same level (2%) was used. For comparison purposes, a sample with a 1% concentration of oil and emulsifier was also prepared. Stability analysis showed no significant changes in droplet size and polydispersity index after stability tests and during 30 days of storage (Table 4). Moreover, all the samples were characterized by a very small droplet size of around 120 nm and low viscosity ($<$ 350 mPas·s), which are desired values of the output parameters.

3.3. Evaluation of antimicrobial activity

3.3.1. Determination of the activity of thyme oil and the emulsion based on the oil against bacteria and yeast-like fungi

The first stage of the study involved determination of the MIC and MBC/MFC values for thyme oil and nanoemulsion based on it (Table 5). The study was carried out for the range of concentrations amounting to 0.078–10 μg/μL. The growth of all bacterial and yeast-like strains, except for *P. aeruginosa* (MIC $>$ 10 μg/μL), was inhibited in the tested concentration range. Thyme oil concentrations: 0.312 μg/μL for *M. luteus* strain, 1.25 μg/μL for *M. catarrhalis, B. subtilis, S. epidermidis, E. coli S. marcescens, P. vulgaris, S. aureus, C. albicans, C. glabrata, C. lipolytica* and *C. tropicalis* strains, and 2.5 μg/μL for *E. faecalis* strain were sufficient to inhibit the growth. Thyme oil had the strongest bactericidal effect (MBC = 1.25 μg/μL) on the following strains: *S. epidermidis, P. vulgaris, S. aureus, M. luteus*. The oil had a slightly weaker effect on *S. marcescens, C. albicans, C. glabrata, C. lipolytica* and *C. tropicalis* strains (MBC/MFC = 1.25 μg/μL). In the highest tested concentration (10 μg/μL), it had a cidal effect on *B. subtilis* and *M. catarrhalis* strains. No cidal effect was observed in the case of *P. aeruginosa, Enterococcus faecalis* strains in the tested concentration range. The MIC and MBC/MFC values for the nanoemulsion prepared based on the oil were also determined. It was found that the nanoemulsion had a static effect at the concentration equal to 0.156 μg/μL on the following strains: *M. luteus, E. coli*, as well as *C. albicans, C. glabrata, C. lipolytica*. The concentration amounting to 0.312 μg/μL inhibited the growth of *M. catarrhalis, B. subtilis, S. epidermidis, S. marcescens, P. vulgaris, E. faecalis, S. aureus*, and *C. tropicalis*. The inhibitory effect of the concentration amounting to 10 μg/μL was only demonstrated in the case of *P. aeruginosa* strain. The cidal effect of the nanoemulsion in the concentration amounting to 0.312 μg/μL was observed in the case of the following bacterial strains: *E. coli, S. aureus, M. luteus*, and yeasts: *C. albicans* and *C. lipolytica*. For the remaining strains, the cidal effect of the oil was as follows: *Proteus vulgaris, C. glabrata, C. tropicalis* (MBC = 0.625 μg/μL), *S. marcescens* (MIC = 5 μg/μL), *Moraxella catarrhalis* (MBC = 10 μg/μL), *S. epidermidis* (MIC = 10 μg/μL), *B. subtilis* (MBC = 10 μg/μL), *E. faecalis* (MIC ⇒ 10 μg/μL), *P. aeruginosa* (MIC ⇒ 10 μg/μL).

3.3.2. Determination of the activity of thyme oil and the emulsion based on the oil against mold and dermatotrophic fungi

The evaluation of effect on mold and dermatotrophic fungi was carried out through the determination of the MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration) values for thyme oil and nanoemulsion based on the oil. The tests were performed in the range of concentrations amounting to 0.078–10 μg/μL in line with the cylinder dilution method on an agar medium. The results obtained are presented in Table 6.

The strongest effect of thyme oil was observed in the case of dermatophytes. The MIC and MFC values of the oil for those fungi were as follows: *M. gypseum* (MIC = MFC = 1.25 μg/μL, *M. canis* (MIC = MFC = 0.625), *T. mentagrophytes* (MIC = MFC = 0.625 μg/μL), *T. violaceum* (MIC = 0.625 μg/μL, MFC = 1.25 μg/μL). A slightly weaker effect of thyme oil was found in the case of mold fungi strains of *S. brevicaulis, A. brasiliensis* species, and the MIC/MFC values amounted to MIC = MFC = 2.5 μg/μL and MIC = 2.5 μg/μL, MFC = 5 μg/μL, respectively. The nanoemulsion had a stronger inhibitory effect than the thyme oil from which it was made. The range of concentrations inhibiting

Table 4. Physicochemical properties of selected nanoemulsions with thyme oil.

Nanoemulsions [%v/v]	Z-Ave [nm]/ PDI after 24 h	Z-Ave [nm]/ PDI after stability tests	Z-Ave [nm]/ PDI after 30 days	Viscosity [mPas·s]
OT-1 (1.0/1.0)	121.3 ± 0.7/ 0.212 ± 0.012	116.0 ± 1.9/ 0.208 ± 0.009	118.8 ± 1.6/ 0.190 ± 0.011	309 ± 10
OT-2 (2.0/2.0)	132.2 ± 1.8/ 0.204 ± 0.006	124.8 ± 2.8/ 0.191 ± 0.004	122.2 ± 1.9/ 0.180 ± 0.001	348 ± 7

Table 5. The MIC and MBC/MFC values for thyme oil and nanoemulsion based on the oil in relation to test yeast-like fungi and bacteria.

Strain	Concentration	Thyme oil	Nanoemulsion based on thyme oil
Moraxella catarhalis	MIC	1.25	0.312
	MBC	> 10	10
Bacillus subtilis	MIC	1.25	0.312
	MBC	10	10
Staphylococcus epidermidis	MIC	1.25	0.312
	MBC	1.25	10
Seratia marcescens	MIC	1.25	0.312
	MBC	2.5	5.0
Proteus vulgaris	MIC	1.25	0.312
	MBC	1.25	1.25
Enterococcus fecalis	MIC	2.5	10
	MBC	> 10	> 10
Staphylococcus aureus	MIC	1.25	0.312
	MBC	1.25	0.312
Micrococcus luteus	MIC	0.625	0.156
	MBC	1.25	0.312
Pseudomonas aeruginosa	MIC	> 10	10
	MBC	> 10	> 10
Escherichia coli	MIC	1.25	0.156
	MBC	1.25	0.312
Candida albicans	MIC	1.25	0.156
	MFC	2.50	0.312
Candida lipolytica	MIC	1.25	0.156
	MFC	2.5	0.312
Candida glabrata	MIC	1.25	0.156
	MFC	2.5	0.625
Candida tropicalis	MIC	1.25	0.156
	MFC	2.5	0.625

MIC – Minimum Inhibitory Concentration [$\mu g/\mu L$], MBC/MFC – Minimum Bactericidal/Fungicidal Concentration [$\mu g/\mu L$].

the growth of the tested fungal strains (MIC) and the fungicidal concentrations (MFC) for the nanoemulsion amounted to 0.312 ⇒ 1.25 μg/μL. The MIC and MFC values were as follows for individual strains: *M. gypseum* (MIC = 0.312 μg/μL, MFC = 0.625 μg/μL), *M. canis* (MIC = 0.312 μg/μL, MFC = 0.625 μg/μL), *T. mentagrophytes* (MIC = MFC = 0.312 μg/μL), *T. violaceum* (MIC = 0.312 μg/μL, MFC = 0.625 μg/μL), *S. brevicaulis* (MIC = 0.625 μg/μL, MFC = 1.25 μg/μL), *A. brasiliensis* (MIC = MFC = 1.25 μg/μL).

Table 6. The MIC, MFC values for thyme oil and nanoemulsion based on the oil in relation to test fungi.

Strain	Concentration	Thyme oil	Nanoemulsion based on thyme oil
Microsporum gypseum	MIC	1.25	0.312
	MFC	1.25	0.625
Microsporum canis	MIC	0.625	0.312
	MFC	0.625	0.625
Trichophyton mentagrophytes	MIC	0.625	0.312
	MFC	0.625	0.312
Trichophyton violaceum	MIC	0.625	0.312
	MFC	1.25	0.625
Scopulariopsis brevicaulis	MIC	2.5	0.625
	MFC	2.5	1.25
Aspergillus brasiliensis	MIC	2.5	1.25
	MFC	5.0	1.25

MIC – Minimum Inhibitory Concentration [$\mu g/\mu L$], MFC – Minimum Fungicidal Concentration [$\mu g/\mu L$].

3.3.3. Assessment of the antiseptic effectiveness of thyme oil, nanoemulsion obtained from it, and commercial preparation

The antiseptic effectiveness was assessed according to the change in the number of cells in the suspension of test microorganisms after 15 minutes and 30 minutes of contact with a potential antiseptic for the following microorganisms: *C. albicans, S. aureus, P. aeruginosa*. The results are shown as the log reduction in the number of viable bacterial cells and fungi after disinfection against the number of cells not exposed to the potential disinfectant. The obtained values are presented in Figure 4. The assessment of the log reduction in the number of cells able to grow is one of the parameters allowing to assess the effectiveness of disinfection. It is recommended in the evaluation of the effectiveness of bactericidal, fungicidal, and sporicidal activity of chemical disinfectants and antiseptic agents (PN-EN 1040:2006; PN-EN 1275:2006). In order to demonstrate the bactericidal activity of chemical disinfectants, the required log reduction needs to amount to 5 or more.

In order to demonstrate the fungicidal activity of chemical disinfectants, the required log reduction needs to amount to 4 or more. In the case of 1% thyme oil, the log reduction of *C. albicans, P. aeruginosa*, and *S. aureus* cells was 3.32–4.69 after 15 minutes, and amounted to 3.96–4.96 after 30 minutes. The nanoemulsion containing 1% thyme oil caused a reduction in the cell count of the above-mentioned strains after 15 minutes and 30 minutes at the level of 1.94–3.21 log and 5.09–6.48 log, accordingly. The nanoemulsion containing 1% thyme oil had bactericidal

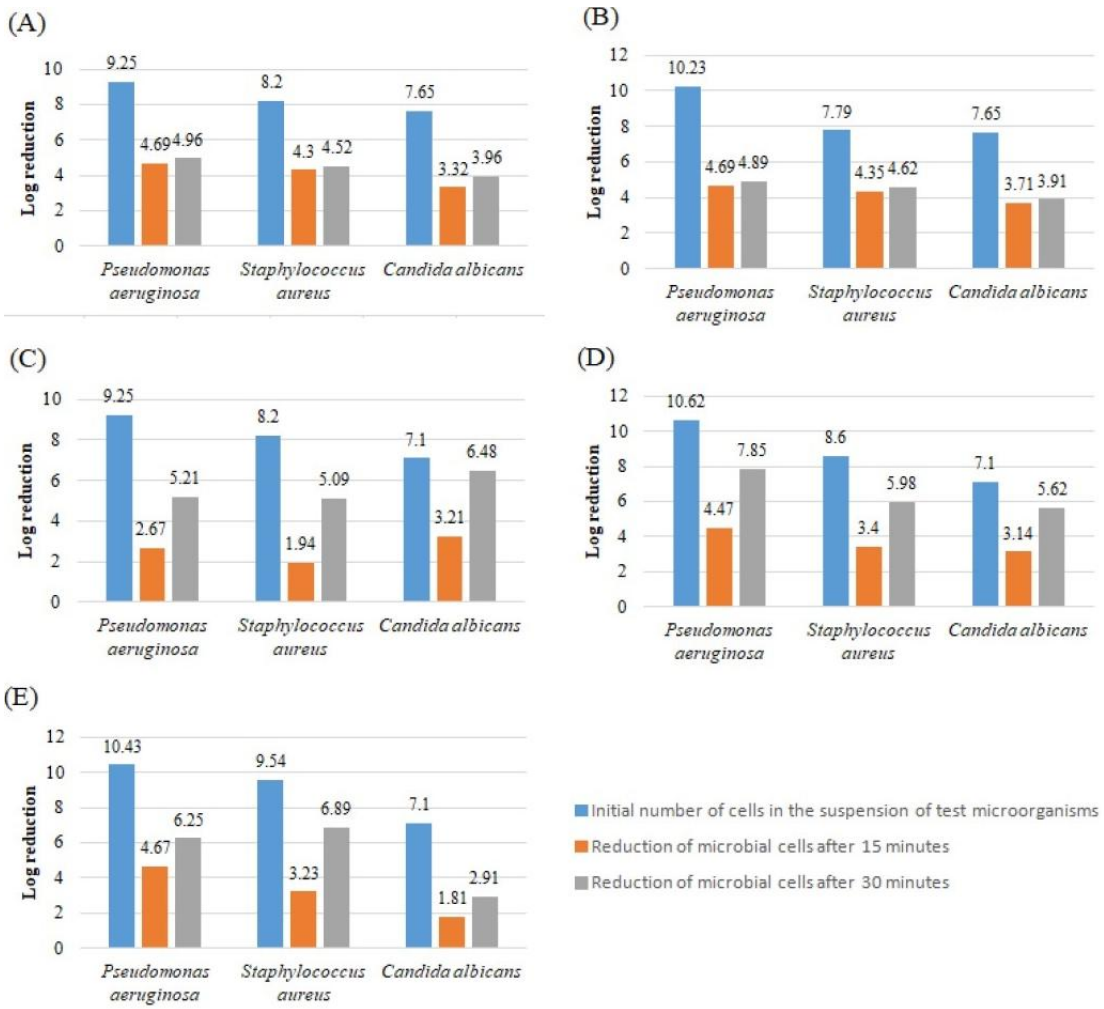

Figure 4. Effect of 1% thyme oil (A), 2% thyme oil (B), 1% thyme oil-loaded nanoemulsion (C), 2% thyme oil-loaded nanoemulsion (D) and N commercial preparation (E) on number of cells in the suspension of test microorganisms.

and fungicidal effect after 30 minutes and caused a decrease in the cell count of *S. aureus* by 5.09 log, *P. aeruginosa* by 5.21 log, and *C. albicans* by 6.48 log. 2% thyme oil caused a reduction of cell counts of the test strains at the level of 3.71–4.69 log after 15 minutes, while the result for the nanoform containing thyme oil of the same concentration was 3.14–4.47 log. The highest cell log reduction value exceeding 5 for bacteria (*S. aureus, P. aeruginosa*) and 4 for yeast-like fungi (*C. albicans*) was obtained after a 30-minute exposure to nanoemulsion based on 2% thyme oil. The values obtained were as follows: 5.98 log for *S. aureus*, 7.89 log for *P. aeruginosa*, and 5.62 log for *C. albicans.*

The commercial preparation caused a decrease in the number of microbial cells amounting to 1.81–4.67 log after 15 minutes. In turn, a 30-minute exposure of the test microorganisms to commercial preparation caused the following levels of reduction: 2.91 log *C. albicans*, 6.89 log *S. aureus*, 6.25 log *P. aeruginosa.*

To sum up: the nanoemulsion based on 1% and 2% thyme oil causes the required reduction $>$ 5 log after 30 minutes of contact with bacterial strains (*S. aureus, P. aeruginosa*) and $>$ 4 log (*C. albicans)* in the case of fungi. The commercial formulation also causes the required reduction $>$ 5 log after 30 minutes in the case of bacterial strains (*S. aureus, P. aeruginosa*). However, it does not have the required fungicidal effect ($>$ 4 log).

4. DISCUSSION

Essential oils, due to their antibacterial properties, have been widely used in the cosmetic, pharmaceutical, and food industries. Literature sources clearly indicate the antibacterial (Aljabeili et al., 2018; Sienkiewicz and Wasiela, 2012; Sienkiewicz et al., 2011) and antifungal (Pina-Vaz et al., 2004; [37]Šegvić Klarić et al., 2007; Zambonelli et al., 2004) effects of thyme oil. However, it should be emphasized that, apart from the active substance itself, the effectiveness of a given preparation also depends on the form of the carrier (Łętocha et al., 2022; Miastkowska et al., 2020). The advantage of nanoemulsion is that it allows to reduce the concen-

tration of the active substance (essential oil) while maintaining biological activity (Al-Assiuty et al., 2019; Miastkowska et al., 2020). In addition, it can improve the solubility of the essential oil and protect against external factors (e.g. oxidation), additionally conditioning the controlled release of the active substance (Velho et al., 2019). Therefore, our research was first based on the selection of the optimal composition of the nanoemulsion and the selection of process parameters. That is why the method of statistical experiment planning (DOE) was used. In the experimental design, the following parameters turned out to be statistically significant: the concentration of essential oil and emulsifier and the ultrasonication time. This is in line with our another report (Rys et al., 2022), where the composition of the peppermint oil nanoemulsion was influenced by the concentration of emulsifier and oil. In this study, an additional parameter determining the best usefulness of the results turned out to be ultrasonication time. This is consistent with the study by (Campolo et al. 2020), which showed a positive effect of the sonication process on the droplet size.

In the present study, these parameters had a significant impact on nanoemulsion particle size and viscosity. On the other hand, in the peppermint oil-loaded nanoemulsion study (Rys et al., 2022), neither the viscosity nor the stability of peppermint oil-loaded nanoemulsions (PNs) depended on any of the input parameters mentioned. The parameter that had a statistically significant impact on the particle size and polydispersity index of nanoformulations was the oil concentration and emulsifier concentration, respectively. These differences may result from different lipophilicity of oils and from the chemical structure and the presence of polar and aromatic groups. (Pauli, 2008) tested the lipophilicity of 25 essential oils. In this study, the lipophilicity of oils increased in the following order: cassia, cinnamon, mustard, parsley, eucalyptus, clove, peppermint, sage, rosemary, star anise, lemongrass, fennel, citronella, cumin, thyme, lavender, pine needles, bergamot, mountain pine, turpentine, angelica, lemon, rose, calamus, and chamomile. Based on this study, peppermint oil is less lipophilic than thyme oil. In turn, the hydrophobicity of the compound significantly affects the particle size distribution (the more lipophilic the oil, the smaller the nanoemulsion droplets) (Jaworska et al., 2013).

Our study confirmed high inhibitory and biocidal activity of thyme oil and nanoemulsion obtained from the oil against a wide spectrum of pathogenic bacterial and fungal microorganisms that cause skin and mucous membrane diseases in humans. It should be noted, however, that the minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC) values for certain test microorganisms were several times lower for the nanoemulsion compared to thyme oil. Particularly high sensitivity to nanoemulsion was observed in the case of bacterial and yeast strains of *S. aureus, M. luteus, E. coli, C. albicans, C. lipolytica, C. glabrata, C. tropicalis* species, for which the MIC and MBC/MFC values amounted to 0.156–0.625 μg/μL. For the same strains, the MIC and MBC/MFC values for thyme oil were 1.25–2.50 μg/μL. Slightly smaller differences in the activity of thyme oil and the nanoemulsion obtained from the oil were found in the case of dermatotrophic and mold fungi. The MIC and MFC values of the nanoemulsion and thyme oil for these fungi amounted to 0.312–0.625 μg/μL and 0.625–5 μg/μL, respectively.

The study also showed the antiseptic effect of nanoemulsions containing 1% and 2% thyme oil on the following microbial strains: *P. aeruginosa*, *S. aureus*, and *C. albicans*. The nanoemulsions caused the cell reduction > 5 log required for antiseptics in the case of bacterial strains (*S. aureus, P. aeruginosa*), and > 4 log (*C. albicans*) in the case of fungi after 30 minutes of exposure. The obtained pathogen cell reduction values were within the following ranges: 5.09–6.48 log (the nanoemulsion containing 1% oil) and 5.62–7.85 log (the nanoemulsion containing 2% oil). However, the nanoemulsion containing 2% thyme oil had a stronger biocidal effect. The obtained cell reduction values for individual test strains were as follows: 5.98 log for *S. aureus*, 7.89 log for *P. aeruginosa*, and 5.62 log for *C. albicans*. The commercial preparation containing silver ions also caused the required reduction > 5 log in the case of bacterial strains (*S. aureus, P. aeruginosa*) after 30 minutes. However, it did not have the fungicidal effect (> 4 log). The cell reduction values after the exposure to commercial preparation for individual strains were as follows: 2.91 log for *C. albican*s, 6.89 log for *S. aureus*, 6.25 log for *P. aeruginosa*. Thyme oil did not show the level of microbial cell reduction required for antiseptics at any of the tested concentrations or contact duration.

The results obtained by us, related to high effectiveness of thyme oil against a wide range of pathogenic microorganisms, have been confirmed in the literature. According to (Sienkiewicz et al., 2011), thyme oil shows high efficiency against clinical bacterial isolates of the genera *Staphylococcus, Enterococcus, Escherichia*, and *Pseudomonas*. According to (Fani et al., 2017), thyme oil in the concentration rage of 16–256 μg/mL also affects pathogens causing oral diseases such as *Streptococcus pyogenes*, *Streptococcus mutans, Candida albicans, Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*. (Omran and Esmailzadeh, 2009) also arrived at interesting conclusions and demonstrated a stronger effect of thyme oil on various *Candida* species than amphotericin B used to treat severe mycosis. The effectiveness of thyme oil and nanoemulsion obtained from it against pathogens causing skin mycoses was mentioned by (Moazeni et al., 2021). A nanoemulsion based on thyme oil showed higher effectiveness in inhibiting the growth of yeast-like and filamentous fungi than the oil itself.

Most authors associate the high effectiveness of thyme oil with its content of phenolic compounds such as thymol and carvacrol (Rota et al., 2008). Thymol and carvacrol were also components of *Thymus vulgaris* tested by us and their content amounted to 35.21 % and 13.27%, respectively. Al-

though the mode of action of these compounds are not clearly understood, it is mostly believed that the hydroxyl group on these two compounds interacts with the cytoplasmic membrane, changes its permeability, and affects the lipid ordering and stability of its bilayer. These results in an increase of proton passive flux across the membrane, their strength, and active transport leading to the disruption of cytoplasmic membrane and leakage of cellular contents (Dhifi et al., 2016; Rota et al., 2008). The antifungal activity of the oil is mostly associated with the direct interaction of thymol, carvacrol, and p-cymene with cytoplasmic membrane ergosterol, which consequently leads to fungal cell membrane disruption and release of the cellular contents (De Lira Mota et al., 2012).

5. CONCLUSION

On the basis of the statistical analysis results, it was found that the concentration of the oil and emulsifier as well as sonication time had the greatest influence on the physicochemical properties of the nanoemulsion. On the basis of approximation profiles, it was concluded that the optimal concentration of the thyme oil in nanoemulsion should amount to 2%. Stability analysis showed no significant changes in droplet size and polydispersity index after stability tests and during 30 days of storage. Biological studies have shown that the nanoemulsions based on thyme oil were characterized by stronger activity against bacteria and yeast-like fungi than pure oil. The MIC values of the nanoemulsion for most of the test strains were 0.156–0.312 μg/μL and MBC/MFC 0.312–0.625 μg/μL. For thyme oil, MIC values ranged from 0.625–2.5 μg/μL, and MBC/MFC 0.625–10 μg/μL. A weaker effect of thyme oil and the nanoemulsion was found for strains of *Pseudomonas aeruginosa* and *Enterococcus faecalis*, for which the MIC and MBC values were 2.5 → 10 μg/μL. Moreover, it was shown that after 30 minutes of contact time, the nanoemulsion based on 2% thyme oil caused a reduction of > 5 log of bacterial strains (*S. aureus, P. aeruginosa*) and > 4 log of fungal strains (*C. albicans*), a level required for disinfectants. In the case of 2% of the oil, the required reduction was obtained only for *P. aeruginosa* (> 5 log) and *C. albicans* (> 4 log). The commercial preparation, containing silver ions, used for comparison purposes, after 30 minutes also caused the required reduction > 5 log test strains of bacteria (*S. aureus, P. aeruginosa*), but did not show the required fungicidal activity (> 4 log) against the strain of *C. albicans*.

To sum up, the thyme oil-based nanoemulsion showed stronger antibacterial and antifungal activity compared to pure oil. Moreover, owing to its antiseptic effectiveness against a wide range of pathogenic microorganisms that cause skin diseases, it may act as a potential skin disinfectant. However, further research is needed to confirm the safety of the obtained formulations after skin application.

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