ORIGINAL ARTICLE

Licorice-garlic-fennel essential oils composite nanoparticles as natural food preservatives

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Abstract

Natural bioactive compounds, such as essential oils, with antimicrobial or antioxidant activity, have received considerable attention recently, especially with rising concerns on the safety of synthetic food preservatives. However, due to their low water solubility and structural stability, their incorporation into water-based food formulations are limited, as is the other lipophilic functional compounds. Moreover, in order to investigate the possible synergistic effects of the most combinations of the essential oils, the aim of this study was to prepare water-dispersible licorice-garlic-fennel essential oil nanoparticles using nanoemulsion systems through a low-energy self-emulsifying technique. The effects of essential oil proportions in the oil phase on nanoemulsion characteristics were also evaluated using a simplex-centroid mixture design, and various empirical models were developed to predict changes in the obtained nanoemulsion characteristics. Finally, multi-goal optimization was applied to obtain the most desired composite nanoemulsions with the least mean particle size, polydispersity index, turbidity, and the greatest antioxidant and bactericidal activity. Based on this optimization analysis, the most desired product was obtained using 39% licorice, 40% garlic, and 21% fennel essential oil as oil phase. The results also confirmed the synergistic effects of selected essential oils towards one another, which the nanoemulsions with two- and three- components oil phase exhibiting higher antibacterial and antioxidant activity than those with a single-component oil phase. The prepared nanoparticles had reasonable physical stability at 5 ± 1 °C during 40 days of storage.

Keywords: *Composite Oil Phase; Essential Oil; Fennel; Garlic; Licorice; Mixture Design; Nanoemulsion.*

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INTRODUCTION

Essential oils, as the mixture of volatile compounds like short-chain fatty acids, polyphenols, terpenes and terpenoids, etc., are originated from various parts of plants, such as buds, flowers, leaves, and bark. They are responsible for the special odor, taste, and flavor of the plant. The health-promoting effects of plant essential oils, such as their antimicrobial and antioxidant activity, have confirmed by various scientific researches. Moreover, due to the toxicity and side effects of synthetic food preservatives, there is an increasing demand for plant essential oil as a safe and effective alternative to commonly used synthetic food, beverages, and cosmetic preservatives [1].

However, essential oils, like other functional lipid compounds are highly volatile, insoluble in water and their structures decompose quickly in the presence of heat and oxygen or light. Moreover, their strong aroma makes some organoleptic undesirability, especially because it has been shown that greater concentrations of essential

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oils are mostly required in food formulations to achieve similar antibacterial or antioxidant effects as commonly used synthetic compounds [2]. Consequently, the researchers have concentrated on the formulation of essential oil mixtures in order to enhance their efficiency and also to incorporate them into nanoemulsions, as oil phase, in order to increase their water-solubility [3,4], structural and chemical stability, effectiveness and thus decrease their effective dosage in various food and beverages formulations.

Glycyrrhiza glabra, commonly known as licorice, has been used as a flavoring agent in medicinal and food constructions for many years. It contains flavonoids, isoflavonoids, glycyrrhizin, glycyrrhetinic acid, chalcones, etc. Licorice extract or essential oil has been used in order to treat hypertension, cough, bronchitis, asthma, ulcers, inflammation, and epilepsy. It can also be used as a sweetening agent in order to cover the unattractive tastes of other food ingredients and organoleptic enhancing properties in various food, beverages, and cosmetic formulations [5]Anarjan N. [3]. *Allium sativum*, known as garlic, has antimicrobial activity against a broad spectrum of bacteria, and fungi. It has been used to treat colds, coughs, tuberculosis, hypotension, vascular disorders and cancers. It has also been used in food systems either as a flavor or digestive aid. The main components of garlic essential oil are allyl polysulfides, such as diallyl sulfide, diallyl disulfide, allyl methyl trisulfide, diallyl trisulfide, allyl methyl disulfide, etc. [6]. *Nigella Sativa* or Fennel essential oil is used generally in medicine and food systems. It has been widely used as an antimicrobial, antioxidant, anticancer, anti-inflammatory, antidiabetic, immunomodulatory, analgesic, spasmolytic, bronchodilator, hepatoprotective, renal protective, gastro-protective agent. The main component of fennel essential oil is thymoquinone [7]. It seems that a synergism can be observed between licorice, garlic, and fennel essential oil and their combination in similar concentrations would have stronger antimicrobial and antioxidant activity as compared to their individual use.

The water insolubility, volatility, and structural instability of essential oil mixture can also be solved by their incorporation into nano-sized carrier systems, like nanoemulsions. Nanoemulsions have drawn special attention as colloidal delivery systems because they can easily be fabricated from food-grade ingredients under quite simple operation conditions, such as mixing, shearing, and homogenization [8, 9]. Nanoemulsions, are thermodynamically unstable but kinetically stable systems that typically consist of oil, surfactant (sometimes co-surfactant/ co-solvent), and water. These systems have small particle size (< 500 nm) with clear and transparent, or only slightly turbid appearance [10-12].

While in previous researches some plant essential oils have been mixed together in order to give the product the highest antibacterial activity against target bacteria, or the highest antioxidant activity using the synergistic effect of essential oils [13, 14], mixing the essential oils as composite oil phase of a nanoemulsion system using mixture design of experiment has not been conducted yet. Hence, the aim of this study was to prepare nanoemulsions with single, binary and ternary combinations of licorice, garlic, and fennel essential oil as oil phase in order to formulate natural food preservers with the highest antimicrobial and antioxidant activity and the smallest mean particle size, polydispersity index (PDI) and turbidity. Special cubic design of experiments was used in experimental design, and thus, the studied characteristics depend absolutely on the ratio of mixture components.

MATERIALS AND METHODS
Materials
Licorice (*Glycyrrhiza glabra*) and fennel (*Nigella sativa*) essential oils were purchased from Najiyan Company (NG, Tabriz, Iran). Garlic essential oil was donated by the Magnolia Company (Tehran, Iran). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Non-ionic surfactants Tween 80 (HLB= 15), and glycerol (HLB 4.5) were purchased from Merck (Darmstadt, Germany). Double distilled water provided by Dr Mojalali Co. (Tehran, Iran). The microbial strains namely, *Escherichia coli* (*E. coli*, PTCC 1276) and *Staphylococcus aureus* (*S. aureus*, PTCC 1431), were donated by the Persian Type Culture Collection (PTCC, Tehran, Iran) and cultured in the laboratory of Food safety (Faculty of Veterinary Medicine, Tabriz Branch, Islamic Azad University, Tabriz, Iran). Mueller Hinton Broth (MHB) medium was purchased from Biolife (Biolife Co., Milan, Italy).

Preparation of nanoemulsions
The self-emulsifying technique was used to prepare the essential oil nanoemulsions. Tween 80

(15%) was first mixed with glycerol (6%) and stirred magnetically (IKA Plate, RCT digital, Deutschland, Germany) for 5 min at a rotating speed of 500 rpm. The essential oils (with various proportions, as seen in Table 1, and the total concentration of 1% w/w) were then added to the Tween 80/ glycerol mixture and stirred for an additional 15 min. The mixture was added drop-wise to a certain volume of distilled water (78%), which was placed in a water bath and stirred with a magnetic stirrer until a homogeneous translucent appearance was obtained. The mixture turned from translucent to fully transparent system after 1 day of equilibration at room temperature [10].

ANALYSIS

Mean particle size and size distribution

The average particle size and size distribution (polydispersity index, PDI) of essential oil nanoemulsions were measured using a zeta-sizer (Nano-ZS, Malvern Instruments, Malvern, UK) and the dynamic light scattering (DLS) technique one day after sample preparation. The measurements were taken at a temperature of 25 °C. Mean particle diameter was reported as z-diameter. All measurements were performed in triplicate [8].

Turbidity

The turbidity of essential oil nanoemulsions was measured using a UV-visible spectrophotometer (PG Instruments Ltd, T70+UVNIS, UK) according to Qian and McClements [15]. The turbidity of samples was calculated using their absorbance at 600 nm. The temperature was set at 25 °C, and deionized water was used as a blank solution.

In-Vitro antioxidative activity (DPPH assay)

Most of the essential oils have antioxidant effects in various food formulations. Thus, the in-vitro antioxidative activity of nanoemulsions can be correlated to the loaded nature of essential oil in formulated nanoemulsions. In which, higher antioxidant activity results from more encapsulation have efficiency of samples. 1 ml of each sample was added to 3.9 ml of DPPH fresh methanolic solution. The absorbance of these solutions and a methanolic solution of DPPH without any nanoemulsions at λ=517 nm was measured using a UV–visible spectrophotometer (PG Instruments Ltd, T70+UVNIS, UK) and coded as A1 and A0, respectively. The radical scavenging activity of samples was calculated using as follows:

$$\text{Scavenging activity (\%)} = (1 - A_1/A_0) \times 100\%, \quad (1)$$

The methanol was used as the blank in measurements. All assays were performed in triplicate [11].

Antibacterial analyses

The agar-well diffusion method was used to evaluate the antibacterial activity of either nanoemulsions or pure and mixed macro-sized essential oils, 500 µl of nanoemulsion samples were pipetted into created wells. The micro-sized essential oils were also diluted in 5% aqueous DMSO in order to give the same concentration as nanoemulsions. DMSO was used as a negative control. The inhibition zones were measured after 24h of incubation of the plates at 37°C. The analysis was carried out in three replicates. The inoculum concentration was approximately 6.5×10^5 CFU.

Morphology

The morphology and microstructure of the optimum nanoemulsion was also determined by transmission electron microscopy (TEM, Hitachi H7500, Japan). One drop of diluted nanoemulsion (1:10) was placed on the film grid, stained by a 1% aqueous solution of phosphotungstic acid, and observed after drying.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The oil phase composition of nanoemulsions or either synergistic or antagonistic effects of essential oil proportions in the oil phase were evaluated using the simplex centroid mixture design of the experiment. The oil phase proportions of the components were then optimized to produce the nanoemulsions with the smallest mean particle sizes, PDI, turbidity, and the highest antioxidant and antimicrobial activity. Unlike the common response surface designs, in mixture design, the independent variables are proportions of the components in a mixture with a certain amount. The common aim of mixture experimentation is to provide a precise estimation of the interaction of the components effects on system behaviors based on a few number of characteristics of the samples. Thus, it is assumed that the responses are influenced solely by the relative proportions of the mixture components, rather than the total volume of the mixture. In this mixture design, the summation of the components should remain constant (when using the actual quantity of

components), and mostly is considered as 1 (when using the fraction of components) or 100 (in using the percentage of components). Accordingly, rising one component proportion allows the proportions of the components to decrease. The proportion of each essential oil (mixture component) varied between 0 (not present in the mixture), and 1 (single component or one component oil phase) [6]. As a result, 12 samples were formulated using a special cubic design configuration, composed of 3 one-component, 3 two-component, and 4 three-component oil phase systems with triplicated center points. In order to estimate the errors, the center point triplication was performed. Therefore, the mixture design was conducted to study the effects of licorice essential oil (x_1), garlic essential oil (x_2), and fennel essential oil (x_3) proportions in oil phase mixture on mean particle size (Y_1), PDI (Y_2), turbidity (Y_3), antioxidant (Y_4), and antibacterial (Y_5) activity.

The oil phase proportions of the components and nanoemulsion characteristics were fitted to a special cubic polynomial equation (Equation 2),

$$Yi = A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 +$$
$$A_{13}X_1X_3 + A_{23}X_2X_3 + AX_1X_2X_3 \qquad (2)$$

where Yi is the predicted response, A_1, A_2, A_3 are the linear effects coefficients, A_{12}, A_{13}, A_{23} are the coefficients of binary or quadratic interaction effects, and A_{123} is the coefficient of the ternary cubic interaction effect of the essential oil components.

The correctness of the fitted models was evaluated using analysis of variance, which is based on gained coefficients of determination (R^2), with R^2 towards 1 corresponds to the most precise models. In suggested models, the interaction terms with p-values less than 0.05 are considered significant in the 95% confident interval, while those with p-value between 0.05 and 0.1 are considered significant in the 90% confident interval [16]. The interactions with a higher F-ratio are more significant than those among the significant terms. After obtaining the best model for each response, the 3D and contour plots were generated to visualize the interaction effects between the components. At last, individual and multiple optimization analyses were applied to obtain the best essential oil composition with the response goal line, such as minimum particle size, PDI, turbidity, maximum antibacterial and antioxidant activity. The models were also verified by comparing the experimental and predicted characteristics of the sample with optimum essential oil compositions. Minitab v.14 statistical packages were used to complete the experimental design, data collection, graph production, and optimization process (Minitab Inc., PA, USA). The experiment design is shown in Table 1 [6,16].

RESULTS AND DISCUSSION
Fitting the models

The mixture design of response surface analysis suggested empirically significant (p-value < 0.05) models for assessing the mean particle size, PDI, turbidity, antioxidant, and antimicrobial activity of essential oil nanoemulsions with various proportions of licorice, garlic and fennel essential oils as oil phase (Table 1). The corresponding F-ratio and p-value offered polynomial model

Table 1. Experimental design and independent levels of parameters.

Sample no.	Licorice essential oil (g)	Licorice essential oil (%v/v)	Garlic essential oil (g)	Garlic Essential oil (%v/v)	Fennel essential oil (g)	Fennel essential oil (%v/v)
1	0	0	0.15	50	0.15	50
2	0	0	0.3	100	0	0
3	0	0	0	0	0.3	100
4	0.3	100	0	0	0	0
5	0.15	50	0.15	50	0	0
6	0.15	50	0	0	0.15	50
7	0.2	66	0.05	17	0.05	17
8	0.05	17	0.05	17	0.2	66
9	0.05	17	0.2	66	0.05	17
10	0.1	33	0.1	34	0.1	33
11	0.1	33	0.1	34	0.1	33
12	0.1	33	0.1	34	0.1	33
Micro-sized licorice essential oil	0.3	0	0	50	0	50
Micro-sized garlic essential oil	0	0	0.3	100	0	0
Micro-sized fennel essential oil	0	0	0	0	0.3	100

Table 2. The p-values and F-ratio of single, binary and ternary interaction terms in suggested models for essential oil characteristics of the nanoemulsions.

Terms	Particle size (nm)		PDI		Turbidity (o.d)		Antioxidant (%)		Antibacterial (mm)	
	P	F	P	F	P	F	P	F	P	F
Linear	0.009	14.01	0.360	1.26	0.001	40.02	0.922	0..08	0.074	4.58
Quadratic	0.027	7.49	0.047	5.61	0.001	35.03	0.333	1.45	0.229	2.03
$x_1 x_2$	0.030	9.02	0.024	10.23	0.004	26.21	0.222	1.94	0.063	5.65
$x_1 x_3$	0.103	3.96	0.057	6.10	0.000	92.32	0.220	1.96	0.373	0.96
$x_2 x_3$	0.059	5.93	0.058	6.00	0.157	2.77	0.223	1.94	0.849	0.04
Special cubic	0.111	3.75	0.779	0.09	0.195	2.23	0.030	9.05	0.035	8.22
$x_1 x_2 x_3$	0.111	3.75	0.779	0.09	0.195	2.23	0.030	9.05	0.035	8.22
R^2	90.50%		87.41%		98.41%		89.85%		91.51%	

[1]Licorice, [2]Garlic and [3]Fennel essential oil

Table 3. The antibacterial activity of macro-sized and nano-sized essential oil, individual or in three-component combination (optimum formulation).

Sample	Antibacterial inhibitory zone against S. aureus (mm)	Antibacterial inhibitory zone against E. coli (mm)
Licorice essential oil	6	5
Garlic essential oil	7	6
Fennel essential oil	5	5
Licorice essential oil nanoemulsion	11	9
Garlic essential oil nanoemulsion	13	10
Fennel essential oil nanoemulsion	11	8
Mixture of licorice, garlic and fennel (39% licorice, 40% garlic and 21% fennel essential oil)	9	9
Mixture essential oil nanoemulsions (oil phase composed of 39% licorice, 40% garlic and 21% fennel essential oil)	15	12

terms, model coefficients, and R² values for each response were shown in Table 2.

The regression models' comparatively high R² (>87%) approves their accuracy in predicting the characteristics of gained nanoemulsions based on their oil phase essential oil proportions (Table 2). As shown in Tables 2 and 3, except for PDI, the single effects of oil phase components had more significant effects on responses (due to their greater F-ratio) than their either binary or ternary interaction effects. Furthermore, with the exception of antioxidant and antibacterial activities, which were significant in 95% and 90% confidence intervals, respectively, all ternary interactions in essential oil proportions were negligible.

Characteristics of essential oil nanoemulsions

The mean particle size of all gained essential oils nanoemulsions was less than 100 nm. Thus, it can be concluded that the self-emulsifying is an efficient technique for size reduction of essential oils into desired nano-scale range. The mean size of nanoemulsions was varied between 4 to 100 nm

by changing the oil phase compositions. Therefore, the oil phase structure has a considerable effect on the particle size of nanoemulsions. The effects of oil phase essential oils proportions on changing of mean particle size of produced nanoemulsions were shown in Fig 1. As can be seen in Table 2, the linear effects of oil phase components were more significant on the mean particle size of samples than their binary and ternary interactions. While the garlic-fennel binary effect was insignificant, the licorice-garlic and licorice-fennel binary effects are significant in 95% and 90% confidence intervals, respectively. Moreover, according to Fig 1 and the negative sign of garlic-fennel regression coefficient, it can be concluded that using a binary mixture of garlic-fennel essential oils in the oil phase can produce smaller nanoemulsions. Furthermore, the particle size of licorice nanoemulsions is smaller than either fennel or garlic nanoemulsions. Equation 3 shows the suggested model in order to predict the mean particle size of produced essential oil nanoemulsions as a function of oil phase proportions of the components. However, the ternary interaction effect was insignificant on
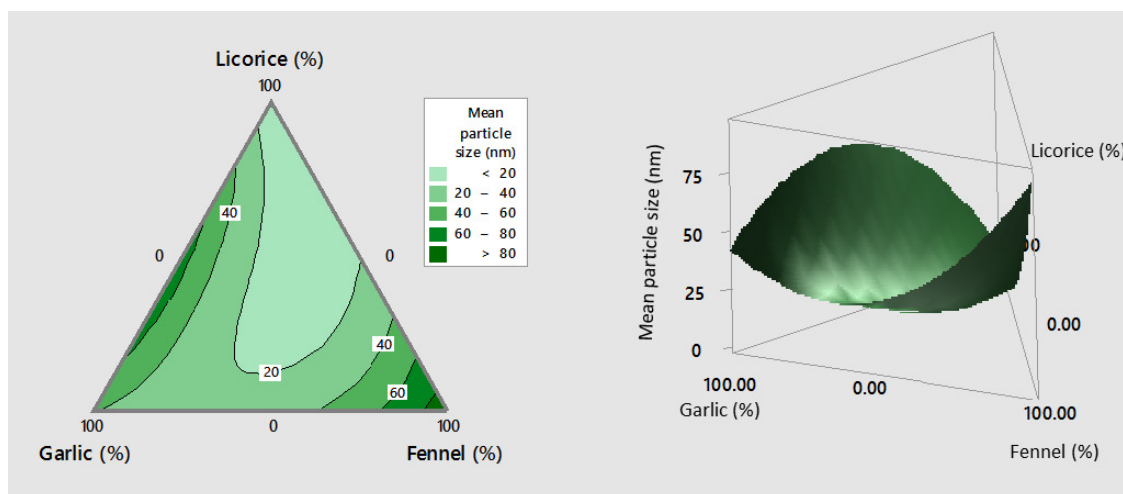
Fig. 1. The effects of oil phase essential oil compositions on essential oil mean particle size nanoemulsions.

particle size of samples, the negative sign of it also indicated that using ternary mixtures of selected essential oils led to the preparation of smaller nanoemulsions. Equation 3 shows the suggested model for the prediction of mean particle size of produced nanoemulsions.

$$\text{Mean particle size} = 5.22755 \, X_1 + 41.6439 \, X_2 + 92.3475X_3 + 179.103 \, X_1X_2 - 118.650X_1X_3 - 145.137 \, X_2X_3 - 627.7X_1X_2X_3 \quad (3)$$

where X1, X2, and X3 are licorice, garlic, and fennel essential oil percentages, respectively.

The insignificant terms can be omitted from equation 3 in order to obtain the reduced and simpler models.

According to the single optimization analysis, the smallest nanoemulsions can be produced using individual licorice essential oil as oil phase.

The oil phase proportions have a major impact on the PDI, homogeneity, or size distribution of the produced nanoemulsions. The analysis of variance for the PDI of samples was shown in Table 2. The findings indicated that the oil phase compositions of nanoemulsions would predict more than 87% of their PDI variations (R^2= 0.87). According to Table 2, the linear or ternary interaction effects of oil phase components had no impact on the PDI of nanoemulsions. Thus, the binary interactions of components, especially licorice-garlic, determines the homogeneity of the system, with using both licorice and garlic in the oil phase, or some

binary combinations of selected essential oils in the oil phase, producing more homogenous nanoemulsions. The PDI of samples was shown in Fig 2. Unexpectedly, the one-component oil phase produced more polydisperse nanoemulsions than the two- or three-component oil phases. Binary mixtures of garlic and licorice essential oil could produce the most desired nanoemulsions.

Equation 4 shows the suggested model for the prediction of PDI of essential oil nanoemulsions.

$$\text{PDI} = 0.639 \, X_1 + 0.768 \, X_2 + 0.958X_3 - 2.33 \, X_1X_2 - 1.799X_1X_3 - 1.784 \, X_2X_3 + 1.174X_1X_2X_3 \quad (4)$$

According to the single optimization analysis, the most homogenous nanoemulsions (the nanoemulsions with the least PDI) can be produced using licorice, garlic, and fennel essential oil with proportions of 50%, 44%, and 6%, respectively.

In general, licorice essential oil is composed of anethole, unsaturated ether aromatics, and glycyrrhizin with a saponin structure. Garlic essential oil contains mainly allyl sulfide compounds, while fennel essential oil contains mostly unsaturated ether aromatics and unsaturated ketone aromatics. The interaction between the active compounds of selected aromatics can manage their steric structures, molecular packing, and their overall polarities [17,18]. As a result of the surface-active action of the licorice essential oil main component, it can enhance the emulsification efficiency of the
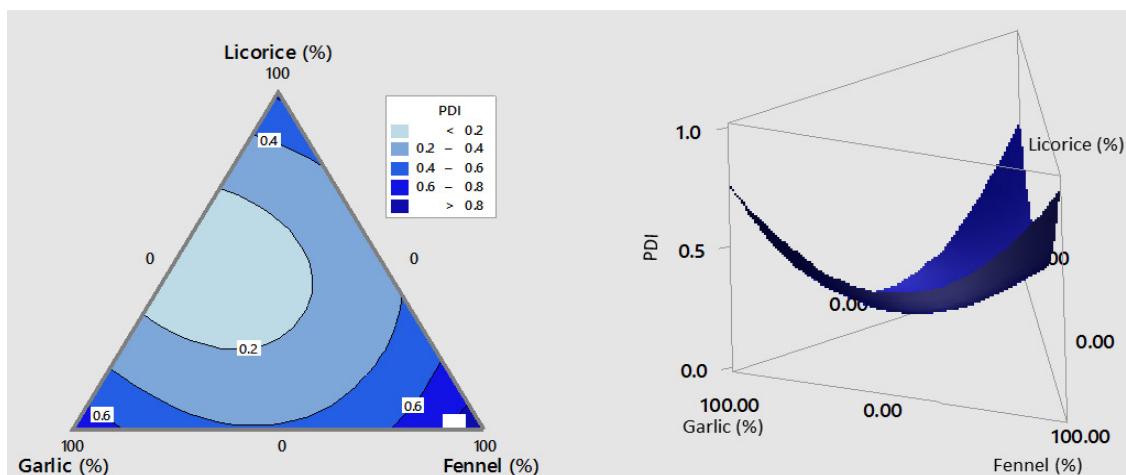
Fig. 2. The effects of oil phase essential oil compositions on PDI of essential oil nanoemulsions.

used emulsifier, and thus, the size reduction and stabilization of nanoparticles will occur more effectively [12].

The garlic essential oil is less polar than licorice and fennel essential oil. Thus, it seems that the garlic essential oil particles break up or their size reduction into nano-range was more difficult than two other essential oil [19]. The nanoparticles would be created if stabilizing molecules made the protective layer at the liquid-liquid boundary through diffusion step. For some essential oils, this protective layer cannot be formed properly due to possible repulsive force between their chemical functional groups and emulsifier molecules. On the other hand, this protective layer can be reinforced if attracting forces arise between the essential oils and emulsifier functional groups. Thus, it seems that the stabilizing capacity for the mixture of licorice and garlic oil increase led to the prevention of ripening, flocculation or aggregation destabilizing mechanisms of nanoemulsions. Thus, since the PDI of samples is determined by the stability of newly broken up nanoparticles, the PDI of systems was decreased in the absence of any destabilizing phenomena [12]. The viscosity of essential oils influences the mean particle size and PDI of their nanoemulsions. The greater size of fennel essential oil nanoemulsions can also be related to its higher viscosity (unpublished data) as compared to garlic and licorice essential oil [20].

The presence of nanoemulsions from the turbidity viewpoint is a good index of their size or physical stability, since less turbid systems are commonly smaller-sized and more stable

nanoemulsion systems. Previous researches concluded that due to their smaller particle size, the less opaque dispersion systems are more stable against ripening, precipitation, and creaming phenomena [21].

The turbidity of essential oil nanoemulsions was altered between 0.0210 and 0.6290. The analysis of variance results for turbidity of samples was shown in Table 2. The turbidity of nanoemulsions was significantly influenced by linear and binary interactions of essential oil proportions in their oil phase. The garlic-fennel binary and all components ternary interactions were insignificant on turbidity changes of samples. The turbidity of prepared nanoemulsions was shown in Fig 3. Fig 3 illustrates that using either binary, or ternary combinations of essential oil with equal proportions, or greater proportions of licorice can produce less turbid and, consequently, more stable nanoemulsions. Unpredictably, the nanoemulsions with one component essential oil are more turbid than the nanoemulsions with two or three combinations of essential oil.

The suggested model for predicting mean particle sizes of produced nanoemulsions is expressed in Equation 5. This model accurately predicts turbidity changes in essential oil nanoemulsions by more than 98% (R^2=0. 9841).

$$\text{Turbidity (o.d.)} = 0.312\, X_1 + 0.220\, X_2 + 0.621 X_3 - 0.868\, X_1 X_2 - 1.629 X_1 X_3 - 0.282\, X_2 X_3 - 1.378 X_1 X_2 X_3 \tag{5}$$
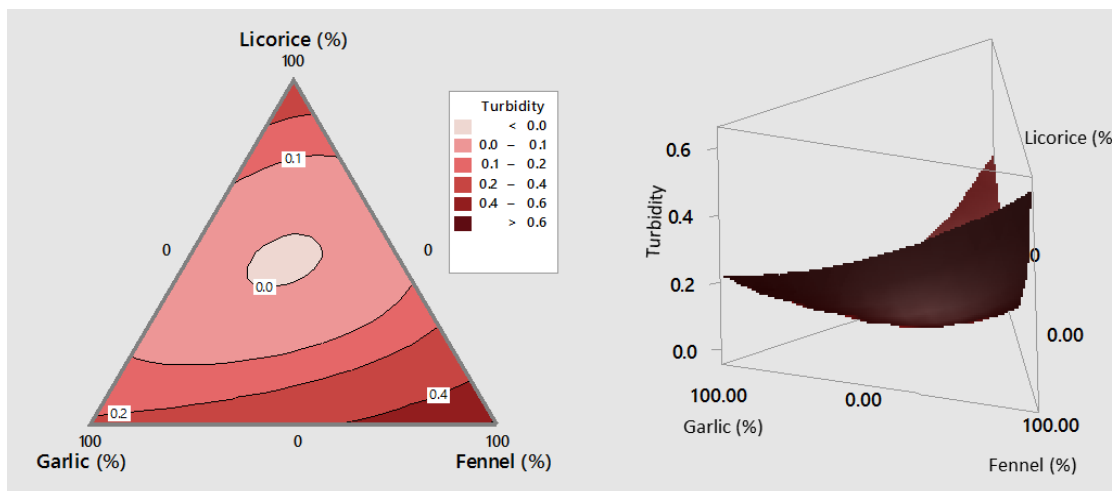
Fig. 3. The effects of oil phase essential oil compositions on turbidity of essential oil nanoemulsions.

It should be noted that the binary garlic-fennel and ternary licorice-garlic-fennel interaction terms in equation 5 could be removed due to their negligible impact on sample turbidity.

The single optimization analysis suggested that the clearest nanoemulsions can be gained at licorice, garlic, and fennel essential oil with proportions of 47%, 28%, and 25%, in the oil phase respectively.

Despite previous researches which found a correlation between the mean particle size and turbidity of nanoemulsions, in the present study there was no association seen between the size and turbidity of samples. This observation can be related to the initial turbid appearance of essential oils and the possible creation of foams during the sample preparation period, due to the presence of surface-active compounds [22].

It was hypothesized that all selected essential oils have antioxidant activity in either macro- or nano-scales, due to their active phenolic and flavonoid ingredients. Moreover, their antioxidant activity can be increased when they are used together. It means that a synergistic effect on antioxidant activity of essential oils can occur when they are used in combination. Thus, the DPPH radical-scavenging activity of proportions of essential oil in the oil phase on the antioxidant activity of nanoemulsions were evaluated to determine either synergistic or antagonistic effects of components towards one another. The DPPH radical-scavenging activity of nanoemulsions was ranged from 64.52 to 88.54%. The analysis

of variance for this response (Table 2) indicated that Licorice-garlic-fennel ternary interaction term was significant, and linear and binary interaction terms were insignificant on antioxidant activities of samples. The antioxidant activity of essential oil nanoemulsions at various oil phase compositions was shown in Fig 4. The results validated the mentioned hypothesis in which synergistic effects were observed in antioxidant activity of essential oil mixtures. According to the analysis of variance results, the ternary interaction effect of essential oils is only the significant term of the suggested model for predicting the antioxidant activity of samples (equation 6). Regarding Fig 4, it can be concluded that the nanoemulsions with a single oil phase had fewer antioxidant activity than those with binary oil phases, and the highest antioxidant activity have resulted in nanoemulsions with a mixture of all three essential oils as oil phase. Thus, combinations of essential oils could increase their antioxidant activity considerably.

The predicting model for antioxidant activity of nanoemulsions can predict more than 89% (R^2=0.8985) change of this response.

$$\text{Antioxidant activity}(\%) = 63.32\,X_1 + 60.99\,X_2 + 61.23X_3 + 31.67\,X_1X_2 + 31.84X_1X_3 + 31.65\,X_2X_3 + 371.87X_1X_2X_3 \qquad (6)$$

All binary interactions can be removed from this model due to their insignificant effects (p-value > 0.05). The single optimization analysis
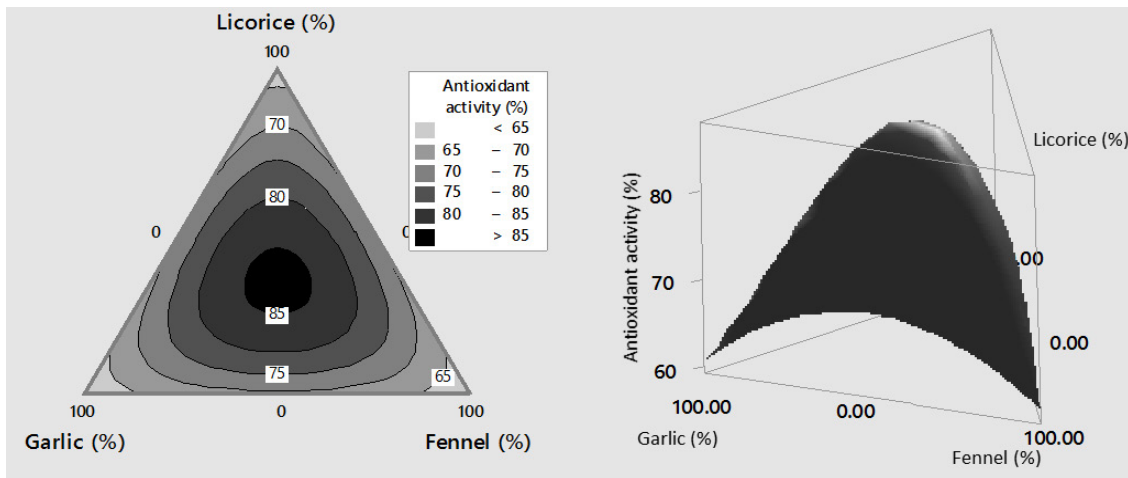
Fig. 4. The effects of oil phase essential oil compositions on antioxidant activity of essential oil nanoemulsions.
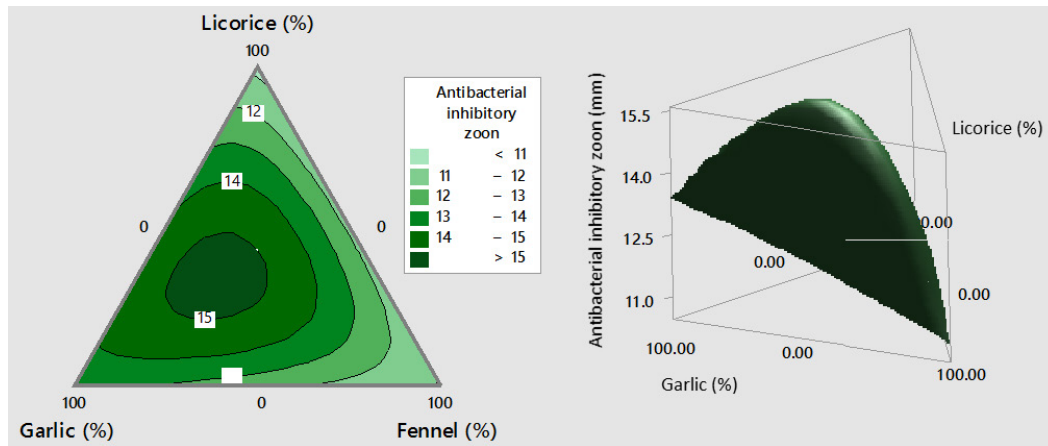

Fig. 5. The effects of oil phase essential oil compositions on antibacterial activity of essential oil nanoemulsions.

predicted that essential oil nanoemulsions with oil phase containing 34% licorice, 33% garlic, and 33% fennel essential oil would have the highest antioxidant activity.

Unlike previous studies that found a reverse correlation between the antioxidant activity of nanoemulsions and their mean particle size, the current research found no such relationship. However, when all three components were used together in the oil process of the collected nanoemulsions, there was a considerable increase in antioxidant activity of the samples. These results are in agreement with previous studies on the antioxidant activity of essential oil mixtures [23].

Because of the chemical structures of the oil phase, essential oil nanoemulsions have exhibited antimicrobial activity, as seen in Fig 5. Thus,

the antibacterial activity of the nanoemulsions formed was evaluated against *S. aureus*, as classic gram-positive bacteria. The analysis of variance (Table 2) indicated that linear terms and licorice-garlic binary interaction terms were significant on antibacterial activity of samples in the 90% confidence interval (p-value< 0.1). The ternary interaction of the essential oils was the most significant term in this response. According to Table 2, the licorice-fennel and garlic-fennel binary interaction terms were insignificant on the antibacterial activity of samples. Similar to antioxidant activity, antibacterial action of samples also improved synergistically when two or three essential oils were combined in the oil process of nanoemulsions. Similar to antioxidant function, antibacterial activity of samples
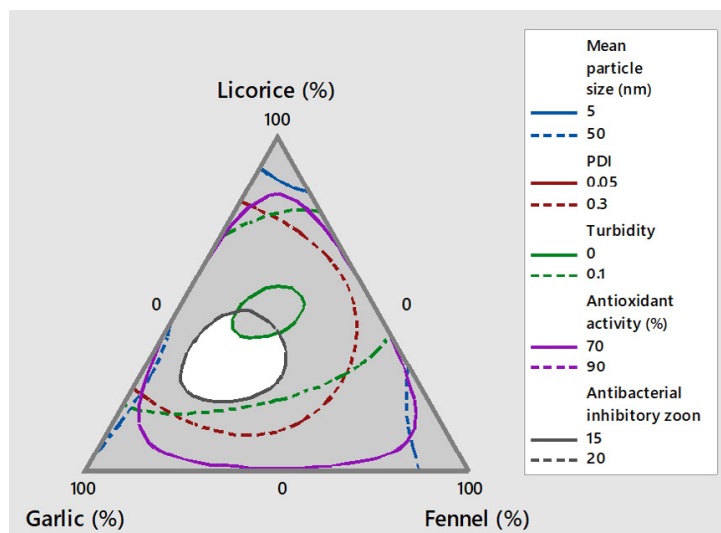
Fig. 6. Overlaid counter plot of licorice, garlic and fennel essential oil proportions in oil phase of nanoemulsions.

improved synergistically when two or three essential oils were combined in the oil process of nanoemulsions. Equation 7 represents the suggested model for the inhibited growth zone of *S. aureus* due to the presence of nanoemulsions.

$$\text{Inhibitory growth zone (against S.aureus)} =$$
$$10.77\, X_1 + 13.40\, X_2 + 10.95 X_3 + 8.34 X_1 X_2 +$$
$$3.43 X_1 X_3 + 0.71 X_2 X_3 + 54.72 X_1 X_2 X_3 \quad (7)$$

The licorice-fennel and garlic-fennel binary interaction terms can be removed from this model due to their insignificant effects. The proposed model can estimate more than 91% (R^2=0.9151) of antibacterial activity changes in essential oil nanoemulsions (Equation 7).

The single optimization analysis predicted that essential oil nanoemulsions with oil phase composed of 34% licorice, 44% garlic, and 22% fennel essential oil would possess the maximum inhibitory zone against bacteria growth and thus the highest antibacterial activity.

It seems that the antimicrobial activity of essential oils is related to their chemical structures in which they attach to the bacterial cell membrane and interpose the normal penetration and transport of components into or outside the cells, and, thus, kill them [10,24].

The antibacterial activity of essential oils against *S. aureus* in either maco-sized or nano-sized form (incorporated into nanoemulsions), alone or in combination were shown in Table 3

and Figs. S1-S8. The results revealed that size reduction of selected essential oils, as well as their combination together in formulations, could increase their antibacterial activity, against either gram-positive or gram-negative bacteria, significantly. It has been reported that general antimicrobial activity of essential oil is related to their phenolic, alcoholic, ketone, and ether compounds, which are mostly presented in the structures of used essential oils, especially when they are used together. Thus, the observed synergistic antibacterial action of essential oils can be explained [25, 26]. The antibacterial activity of nano-sized essential oils, against *S. aureus* and *E. coli* could be increased 1.85 and 1.56 times, respectively, as compared to macro-sized ones.

Previous studies have also shown a long-lasting antibacterial activity for nano-sized essential oils as compared to their macro-sized form on *E. coli, B. subtilis*, and *S. aureus* due to controlled release action of the selected delivery systems [27].

Multiple optimizations
Both graphical and numerical multiple optimizations were applied to find the best oil phase composition, leading to the production of essential oil nanoemulsions with the smallest particle size, polydispersity index, turbidity, and the highest antioxidant and antimicrobial activity. The overlaid counter plot for graphical optimization has seen in Fig. 6. The white color was labeled as the best organic phase proportions of

Table 4. The experimental and predicted values for characteristics of essential oil nanoemulsions.

Sample	Mean particle size (nm)		PDI		Turbidity		Antioxidant activity (%)		Antibacterial inhibitory zone (mm)	
	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.
1	37.33	30.7115	0.485	0.416935	0.372	0.349971	70.34	69.0229	12	12.3527
2	44.92	41.6439	0.794	0.768149	0.234	0.219713	61.64	60.9892	13	13.4036
3	95.69	92.3475	1.000	0.957785	0.629	0.621258	61.90	61.2337	11	10.9491
4	4.38	5.2275	0.643	0.638785	0.300	0.312349	64.54	63.3247	11	10.7673
5	70.64	68.2115	0.151	0.120935	0.051	0.049062	71.94	70.0739	14	14.1709
6	21.62	19.1251	0.395	0.348571	0.055	0.059607	72.12	70.2384	12	11.7164
7	13.65	16.8784	0.142	0.226924	0.065	0.037633	71.23	77.4084	13	13.5782
8	17.62	33.4184	0.233	0.431924	0.208	0.240905	71.83	76.3612	13	13.0327
9	17.28	32.8793	0.143	0.292833	0.051	0.103542	71.74	76.2248	16	14.6691
10	15.38	13.7490	0.216	0.174720	0.039	0.024653	88.83	86.1943	15	15.1200
11	26.28	13.7490	0.351	0.174720	0.021	0.024653	88.52	86.1943	15	15.1200
12	16.90	13.7490	0.174	0.174720	0.043	0.024653	88.83	86.1943	15	15.1200

Exp.: Experimental, Pred.: Predicted

the components, resulting in the best composite essential oil nanoparticles. According to Fig 6, the optimum oil phase proportions were found in the three-component oil phase area (inside the triangle), with quite equal proportions of licorice and garlic and fewer proportions of fennel essential oil.

The proposed optimum oil phase proportions zone successfully supports the research hypothesis that the antioxidant and antimicrobial activity of essential oils can be enhanced synergistically when used together as mixtures of essential oils in formulations.

The numerical optimization also resulted in an oil phase composed of 39% licorice, 40% garlic, and 21% fennel essential oil, which can provide the most desirable nanoemulsions with minimum particle size, PDI, turbidity, and maximum antioxidant and antibacterial activity. The corresponding predicted response values under these optimum oil phase proportions of the components were 31.35 nm for mean particle size, 0.136 for PDI, 0.003 for turbidity, 84.51% for antioxidant activity, and 15 mm antibacterial inhibitory zone.

According to Table 3, the mixing of selected essential oils in optimum proportions (39% licorice, 40% garlic, and 21% fennel essential oil) could enhance their antibacterial activity 1.45 and 1.65 times in macro-sized form, and 1.27 and 1.30 times in nano-sized form, against *S. aureus* and *E. coli,* respectively.

Validations of the model

The experimental data and predicted ones (Table 4) for each response were compared using paired t-test. The obtained p-value (p-value=1)

showed that there was no significant difference between the experimental and predicted data. Thus, all of the suggested models were suitable for predicting the characteristics of the produced nanoemulsions.

Moreover, the best essential oil nanoemulsions were produced in triplicates at the desired oil phase proportions (39% licorice, 40% garlic, and 21% fennel essential oil), and their features were measured (mean particle size=35±5nm, PDI=0.122±0.043, Turbidity=0.003±0.001, Antioxidant activity =86.90±2.5% and Antibacterial inhibitory zone=16±1 mm). A t-test statistical analysis was performed to evaluate the similarities between measured characteristics at the 95% confidence level (p-value < 0.05). The obtained p-values (p-value =1.00) for all responses approved the similarities of predicted and measured data. Therefore, the models were validated and their correctness was re-confirmed.

Storage studies

The size distribution of optimum essential oil nanoemulsions was shown in Fig 7a. The characteristics of these optimum samples after 40 days storage at 5±1°C were; mean particle size= 38.10±8.8nm, PDI=0.130±0.0555, Turbidity=0.004±0.0005, Antioxidant activity=80.00±3.89%, and Antibacterial inhibitory zone=16±1 mm. Except for antioxidant activity, which decreased during 40-day storage, no significant differences in the characteristics of fresh and stored essential oil nanoemulsions (p-value=1) were found using a comparison t-test. Thus, the physical stability of nanoemulsions can be confirmed.
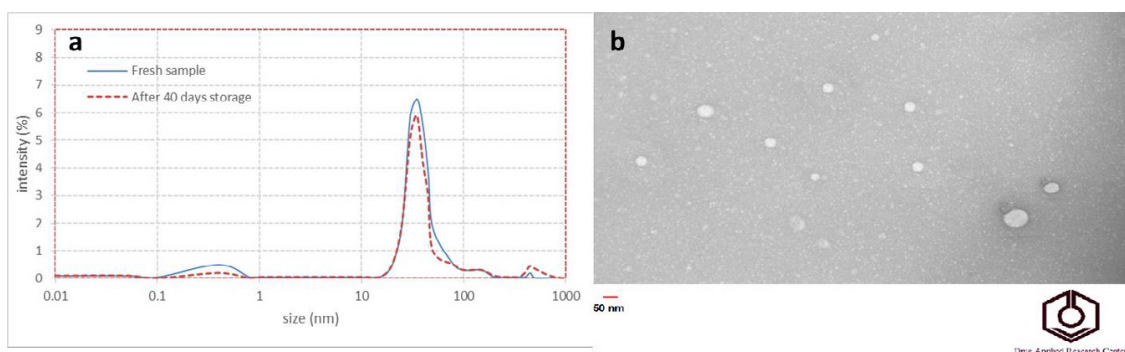
Fig. 7. a. The size distribution (fresh and after 40-days storage at 5±1 °C), and b. TEM image of optimum essential oil nanoemulsions (oil phase: 39% licorice, 40% garlic and 21% fennel essential oil, fresh sample).

Morphology

The morphology of essential oil nanoemulsions with optimum essential oil proportions in the oil phase (39% licorice, 40% garlic, and 21% fennel) was also characterized by electron microscopy (TEM) technique (Fig 7b). The essential oil nanoemulsions with optimum oil phase composition were relatively in spherically-shaped particles. The prepared optimum sample nanoparticles were quite homogeneous, with relatively similar sizes less than 50 nm (approximately 35 nm), in agreement with particle size analysis findings using light scattering theories.

CONCLUSION

The licorice-garlic-fennel essential oil composite nanoemulsions were successfully prepared through self-emulsification technique, in mean particle size less than 100 nm. The oil phase proportions of the components were optimized aimed to get the composite essential oil with minimum particle size, PDI, turbidity and maximum antibacterial and antioxidant activity using a simplex centroid design of mixture analysis. The effects of oil phase compositions proportions on characteristics of obtained nanoemulsions were also evaluated and empirical significant (p-value < 0.05) models were also suggested calculating the studied characteristics of nanoemulsions. It was concluded that the size reduction of essential oil into nano-range and mixing them simultaneously, could provide the particle size reduction and synergism benefits for the product and significantly improve their biological and physicochemical properties. Based on the defined goals, the nanoemulsions with

39% licorice, 40% garlic and 21% fennel in the oil phase exhibited the most desired features. Consequently, a three-component oil phase with quite similar proportions of licorice and garlic and smaller proportions of fennel yielded the optimum licorice-garlic-fennel essential oil composite nanoemulsions. The antibacterial activity of nano-sized essential oil against *S. aureus* and *E. coli* could be increased 1.85 and 1.56 times, respectively, as compared to macro-sized ones. Besides, the mixing of selected essential oils could enhance the antibacterial activity of samples, 1.45 and 1.65 times, in a macro-sized form, and 1.27 and 1.30 times, in a nano-sized form, against *S. aureus* and *E. coli,* respectively. The prepared nanoparticles had reasonable satisfactory physical stability at 5 ± 1 °C during 40 days storage. The formulated optimum licorice-garlic-fennel essential oil composite nanoemulsions can be successfully used in various food and beverage formulations as natural health promoting preservative agents.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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