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RESEARCH ARTICLE

Effects of hydrophilic and lipophilic emulsifier concentrations on the characteristics of Germander essential oil nanoemulsions prepared using the nanoprecipitation technique

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Abstract

The Germanders (*Teucrium polium* L.) essential oil exhibits antioxidant and bactericidal activities against a wide range of microorganisms; however, its water insolubility, susceptibility to environmental stresses, and intense flavors limit its uses in food formulations. As a solution, in the present study, nanoemulsions of Germanders (*Mentha pulegium*) essential oil were prepared using a bottom-up nanoprecipitation technique. A central composite design based on the response surface methodology was implemented to investigate the effects of selected lipophilic and hydrophilic emulsifier concentrations. The proposed second-order polynomial models, with relatively high coefficients of determination, could efficiently predict alterations in response parameters due to emulsifier concentrations. The results revealed that both lipophilic and hydrophilic emulsifiers had significantly affected all characteristics of the synthesized essential oil nanoemulsions. Multi-goal optimization analysis suggested that 7.8% and 4.8% concentrations of Span 80 and Tween 80, respectively, could yield the most desirable Germanders essential oil nanoemulsions, with a mean particle size of 78.56 nm, PDI of 0.1722, DPPH radical scavenging of 83.69%, *Staphylococcus aureus* and *Salmonella enterica* growth inhibition zones of 10.5 mm and 12.7 mm, respectively. The validity of the models was confirmed by the absence of substantial variations between experimental data and modeling results. While the prepared Germander essential oil nanoemulsions demonstrated acceptable physical properties, they exhibited relatively limited chemical stability during storage at 5°C for 30 days.

Keywords

Emulsifiers; food preservative; nanoemulsions; germanders essential oil; physicochemical properties

Introduction

The Germanders (*Teucrium polium* L.) essential oil is primarily composed of various aldehyde, ketone, alcohol, acid, and terpene compounds. Like many other essential oils, Germander essential oil exhibits antioxidant and bactericidal activities against a wide range of microorganisms (1, 2). However, similar to other functional lipid compounds, essential oils have limited water solubility and structural stability. Consequently, water insolubility, susceptibility to environmental stresses, and their intense flavors can restrict the use of essential oils in various food formulations as natural preservatives (3).

Recently, nanotechnology has provided an efficient solution to the challenges of using essential oils in food formulations by reducing their size to nano-ranges. It has been previously established that lipid-based organic compound nanoparticles can enhance water solubility (dispersibility), chemical stability, and bioactivities compared to their macro-sized counterparts (4-9). Consequently, by producing nanoparticles, the accuracy of the models was proven by the absence of significant variations between experimental data and modeling results. The large surface area-to-volume ratio of nanoparticles makes them ideal candidates for composite materials, chemical reactions, drug delivery, and energy-saving applications (10). Improving the properties of bioactive compounds, including solubility, biological uptake, electro-optical, and other physical properties, can be achieved by reducing their particle sizes to the nanometer range (9, 11, 12).

Size reduction of organic compounds can be achieved through either high or low-energy techniques, each with its advantages and drawbacks (9, 13). The most suitable size reduction technique is typically selected based on the available facilities, materials, active compound chemical structure, and desired product characteristics. Principally, the size distribution of nanoparticles governs their physical stability. To achieve the highest physical stability over time, the particle size distribution should be as narrow as possible (9, 12, 13).

According to this theory, the only effective forces in nano-sized colloidal systems are van der Waals forces and electrostatic repulsion. The repulsive forces among particles result from the electrostatic repulsion forces on the surfaces of nanoparticles coated by emulsifier and stabilizer materials. This electrostatic repulsive force ensures the physical stability of nanoparticles, and is proportional to the net zeta potential values of nanoparticles. Thus, nanoparticles with high net zeta potential values have greater physical stability (11, 14). Furthermore, the lower conductivity of nanoparticles reduces their ripening and increases their physical stability (15).

Incorporating essential oils into nanoemulsions can be a straightforward technique for producing nano-sized essential oils. The low-energy solvent-displacement method is one of the most common procedures for preparing nanoemulsions. A limited number of approved emulsifiers for food formulations poses a challenge in creating food nanoemulsions (3, 12). Tweens and Spans, which are food-grade nonionic emulsifiers with low toxicity, biocompatibility, non-irritability, and a high capability to form nanoemulsions, can be used to prepare essential oil nanoemulsions for food formulations (16). Therefore, in the current study, Germanders essential oil nanoemulsions were prepared using Tween 80 and Span 80 as emulsifiers through the solvent-displacement technique. To the best of author's knowledge, no study has been conducted using two emulsifiers simultaneously to create nanoemulsions. The objective of this research was to find the optimal concentrations of both Tween 80 and Span 80 as lipophilic and hydrophilic emulsifiers to develop nanoemulsions with the smallest average particle size, size distribution, conductivity, and the highest net zeta potential, antibacterial and antioxidant activities. These nanoemulsions can then be used in various food formulations as natural preservatives.

Materials and Methods

Materials

The Germanders essential oil was purchased from Najian Company (NG, Tabriz, Iran). DPPH (2,2-diphenyl-1-picrylhydrazyl), Tween 80 (Polyoxyethylene (20) sorbitan monolaurate), and Span 80 (Sorbitan monolaurate) were obtained from Merck (Darmstadt, Germany). All solvents were acquired from Dr. Mojalali co. (Tehran, Iran). The bacterial strains (*Staphylococcus aureus*, PTCC-1112, and *Salmonella enterica*, PTCC-1709) in the form of lyophilized ampoules were sourced from the Iranian Research Organization. All microbial culture media were also procured from Merck (Darmstadt, Germany).

Preparation of Germander essential oil nanoemulsions

To prepare the Germanders nanoemulsions, both aqueous and organic phases were prepared. The organic phase was obtained by dissolving 1% Germander essential oil and Span 80 (in concentrations according to experimental design, Table 1) in 10 mL of acetone. To prepare the aqueous phase, Tween 80 (in concentrations according to experimental design, Table 1) was dissolved in 40 mL of deionized water. Subsequently, the organic phase was added dropwise to the aqueous phase using a magnetic stirrer at 100 rpm. The mixing of phases continued for an additional 15 min. afterward, acetone was removed from the system using a rotary evaporator at reduced pressure (Heidolph, Germany) at 50 rpm, 40°C, and 0.4 atm. After eliminating the solvent, the essential oil nanoemulsions were obtained and stored at 2°C for characterization. All experiments were performed in duplicate to avoid experimental errors, and standard deviations were presented in Table 1.

Analysis

Mean particle size, polydispersity index (PDI), the zeta potential, and conductivity

The mean particle size and PDI of the produced nanoemulsions were determined using a particle zeta-sizer (Malvern Instruments Ltd., Nano ZS, UK) based on the dynamic light scattering method. The PDI ranged from 0 to 1, with values closer to 0 representing a narrow size distribution and greater homogeneity in the system. The zeta potential and conductivity of the nanoemulsion were also measured using the aforementioned instrument by determining the electrophoretic mobility distribution of nanoparticles based on the Smoluchowski equation, which utilizes the measured particle velocity. Prior to analysis, the samples were diluted 1:10 in deionized double-distilled water, and their pH and temperatures were set at 7.0 and 25°C, respectively (3).

Table 1. Experimental design, independent and dependent variables in the preparation of Germanders essential oil nanoemulsion.

Sample No.	Tween 80	Span 80	HLB	Mean particle size(nm)	PDI	zeta-Potential (-mV)	Conductivity (mS.cm)	Turbidity (od)	Antioxidant (%)	Growth inhibition zone against *Staphylococcus aureus* (mm)	Growth inhibition zone against *Salmonella enterica* (mm)
1	9	3	12.32±0.45	47.27±2.25	.521±0.022	2.37±0.62	0.201±0.061	0.036±0.021	85.05±2.35	10.12±0.46	12.23±0.23
2	9	3	12.32±1.01	50.46±3.25	0.162±0.038	3.80±0.01	0.205±0.088	0.037±0.015	84.67±2.45	10.52±0.33	12.21±0.31
3	7	5	10.43±0.58	56.55±1.65	0.722±0.025	4.26±0.22	0.207±0.052	0.023±0.003	86.44±3.25	10.23±0.34	12.81±0.43
4	11	1	14.21±0.22	170.78 ±4.26	0.598±0.043	3.64±0.93	0.161±0.021	0.077±0.002	71.87±2.87	10.84±0.27	12.00±0.32
5	12	3	12.86±0.46	310.75 ±8.65	0.334±0.002	5.04±0.21	0.185±0.063	0.108±0.003	57.52±3.10	10.00±0.34	12.51±0.41
6	9	3	12.32±0.35	44.85±1.65	0.288±0.014	3.37±0.39	0.200±0.092	0.036±0.005	84.94±2.25	10.91±0.36	12.00±0.28
7	6	3	11.43±1.25	110.24 ±3.69	0.294±0.005	4.06±0.27	0.216±0.013	0.027±0.011	84.90±2.38	10.25±0.46	12.95±0.42
8	7	1	13.78±1.02	51.67±3.12	0.163±0.043	3.35±0.051	0.187±0.056	0.022±0.001	89.56±3.24	10.76±0.36	12.92±0.35
9	9	3	12.32±2.05	58.52±2.12	0.160±0.012	2.69±0.62	0.201±0.058	0.038±0.008	83.69±2.65	10.22±0.45	12.82±0.31
10	9	0	15.00±0.89	47.56±1.56	0.333±0.065	2.36±0.54	0.158±0.083	0.037±0.003	85.83±3.54	10.51±0.54	12.51±0.45
11	9	6	10.72±0.95	49.46±0.58	0.301±0.062	3.38±0.33	0.196±0.032	0.036±0.039	82.08±3.85	10.00±0.36	12.91±0.35
12	11	5	11.62±1.52	175.15±10.23	0.168±0.023	4.59±0.52	0.193±0.043	0.077±0.012	68.18±2.56	10.91±0.39	12.54±0.24
13	9	3	12.32±1.85	53.61±1.87	0.163±0.031	4.97± 0.23	0.192±0.055	0.037±0.014	83.88±3.25	11.12±0.42	12.70±0.42

Turbidity

In this study, the absorbance of samples at 600 nm was measured using a UV–Vis spectrophotometer (T70+UV/VIS Spectrometer, PG Instruments Ltd, England) and recorded as the turbidity of the products. Deionized water was used as a blank solution (7).

Antioxidant activity

The *in vitro* antioxidant efficacy of essential oil nanoemulsions was obtained using the DPPH radical scavenging technique. DPPH can donate a hydrogen atom to antioxidant compounds and be neutralized. The reduction in the DPPH concentration in the system was then observed by a decrease in color intensity (from violet to yellow). 2 mL of the nanoemulsions was added to 2 mL of methanolic DPPH solution (0.1mM DPPH) and left for thirty minutes at room temperature (25±2°C). The absorbance of these mixtures was measured at 520 nm using a UV–Vis spectrophotometer (PG Instruments Ltd, England). The antioxidant or DPPH-scavenging activity of samples was estimated using the following equation:

Scavenging activity (%) = $(1 - A_1/A_0) \times 100\%$ ....(Eqn. 1)

In this equation, A_1 corresponds to the absorbance of the tested sample and A_0 to that of the blank controls. The blank control used a methanolic solution of DPPH without nanoemulsion samples (3).

Antibacterial activity

The antibacterial activity of the samples was assessed using the agar well diffusion method. First, the cultured bacterial strains were diluted with normal saline to achieve a 0.5 McFarland standard solution. The diluted bacterial strains (*Staphylococcus aureus* or *Salmonella enterica*) were inoculated using sterile cotton swabs onto Muller Hinton Agar. Wells were created using a sterile Pasteur pipette on each agar plate. 30 µl of each sample was placed in its respective well. The plates were incubated for 24 hr at 37°C before measuring the diameters of the growth inhibition zones (17).

Gas chromatography-mass spectrometry (GC-MS)

The chemical composition of Germander essential oil nanoemulsions was determined using the gas chromatography technique (Agilent 6890N, US) coupled with a mass spectrometer detection device (Agilent 5973N, the United States). The essential oil used in this experiment was evaporated with helium and then injected into a column that was 30 m long, 0.32 mm wide and had a stationary-phase particle size of 0.25 µm (5MS-HP Agilent, US). The oven temperature was increased from 45°C to 250°C by adding 5°C per minute. Data analysis and instrument control were performed using Agilent's G1701DA MSD ChemStation, version D.00.00.38 (17).

Experimental design and statistical analysis

The concentrations of emulsifiers, namely, Tween 80 and Span 80, were considered as independent parameters, while the mean particle size, PDI, zeta potential, conductivity, turbidity, antioxidant activity, and antibacterial ability of obtained essential oil nanoemulsions against *Staphylococcus aureus* and *Salmonella enterica*, were studied as response variables. The experimental design, data analysis, model development, graph preparations, and optimization process were performed based on the central composite design of the response surface method using Minitab v. 14 statistical packages (Minitab Inc, State College, PA) (18).

The ability of the models to predict the response variables was evaluated by their calculated coefficient of determination (R^2). The statistical significance of each regression term was also assessed using their obtained p-value and F-ratio through ANOVA analysis. It should be noted that the proposed models are only valid within the studied ranges of variables (19-21).

Results and discussion

Tweens and Spans are efficient nonionic emulsifiers in formulating various food, pharmaceutical, and cosmetic nanoemulsions. It is possible to prepare a wide range of stable O/W and W/O nanoemulsions using proper combinations of these surface-active compounds. Thus, this study aimed to obtain the optimal combinations of Tween 80 and Span 80, as well-known Tween-Span grouped emulsifiers, in the preparation of the most desirable Germanders essential oil nanoemulsions. Since the hydrophilic-lipophilic balance (HLB) values for pure Tween 80 and Span 80 are 15 and 4.3, respectively, the HLB values for emulsifiers in each sample were also calculated and recorded (Table 1, $HLB_{(total)}=\Sigma HLB_i (x_i)$), to find a valid relationship between HLB and characteristics of obtained nanoemulsions (Table 1). Since hydrophilic emulsifiers have HLB values above 10 and the HLB of lipophilic ones lies between 1 and 10, it can be concluded that in the current research, all combinations of Tween 80 and Span 80 used in the preparation of nanoemulsions were hydrophilic (16, 22-24).

Mean particle size and PDI

Germanders essential oil nanoemulsions were effectively synthesized using the solvent-displacement technique. The properties of the Germander nanoemulsions that were manufactured using various concentrations of Tween 80 and Span 80 are shown in Table 1. As can be observed in Table 1, the mean particle sizes of the obtained Germander essential oil nanoemulsions ranged from 47.27 to 310.75 nm.

The ANOVA analysis results for determining the significance of independent variables on the mean particle size of nanoemulsions in terms of linear, quadratic, or interaction effects can also be seen in Table 2. According to ANOVA analysis, the mean particle size of nanoemulsions was affected only by the Tween 80 concentration. Therefore, unlike Tween 80, any changes in the concentration of Span 80, the fat-soluble emulsifier, did not alter the mean particle size of the products.

Equation 2 represents the proposed polynomial model derived from multiple regression analysis, which predicts the changes in the mean particle size of samples based on the emulsifier concentrations. Equation 2 is a reduced model with insignificant terms removed.

$$\text{Mean particle size (nm)} = 1147.2 - 275.1\ \text{Tween 80} + 16.992\ \text{Tween 80}*\text{Tween 80} \quad \text{................ (Eqn. 2)}$$

The R^2 value for this model is 89.34%. This means the model can predict more than 89% of the mean particle size values of the obtained nanoparticles.

The effects of Tween 80 concentrations can be seen in Fig. 1. It is evident that increasing the Tween 80 concentrations up to a certain point (approximately 8%) results in a decrease in the mean size of the nanoemulsions. Further increases in Tween 80 concentration cause an increase in the nanoparticles' diameters to a certain extent. The turning point or the lowest point in the changes in the average size of particles in the nanoemulsions with respect to the Tween 80 concentration can also be confirmed by examining the signs of the linear and quadratic effects of this parameter. The negative sign of the linear coefficient and the positive sign of the quadratic coefficient indicate that increasing Tween 80 leads to a decrease in the mean particle size of nanoemulsions. However, further increases in Tween 80 concentration have the opposite effect.

Table 2. The p-value and F-ratio of Germanders essential oil nanoemulsion characteristics' changes by emulsifiers' concentrations.

Source		Mean particle size	PDI	Conductivity (mS/cm)	Turbidity (od)	Antioxidant activity (%)
Model	p-value	0.000	0.000	0.000	0.000	0.000
	F-ratio	118.42	57.55	48.37	54.36	202.74
Linear effects	p-value	0.000	0.000	0.000	0.000	0.000
	F-ratio	133.17	126.69	82.89	109.07	364.64
X_1 (Tween 80)	p-value	0.000	0.000	0.000	0.000	0.000
	F-ratio	266.21	63.03	63.80	218.15	710.55
X_2 (Span 80)	p-value	0.720	0.000	0.000	0.922	0.003
	F-ratio	0.14	190.35	101.98	0.01	18.73
Quadratic effects	p-value	0.000	0.002	0.000	0.000	0.000
	F-ratio	162.89	16.43	36.83	26.84	142.18
X_1^2 (Tween 80×Tween 80)	p-value	0.000	0.003	0.959	0.000	0.000
	F-ratio	313.14	18.89	0.00	51.97	278.15
X_2^2 (Span 80×Span 80)	p-value	0.263	0.004	0.000	0.295	0.776
	F-ratio	0.18	18.26	72.53	1.28	0.09
Interaction effect	p-value	0.982	0.259	0.165	0.985	0.783
	F-ratio	1.16	1.51	2.41	0.00	0.08
X_1X_2 (Tween 80×Span 80)	p-value	0.0.982	0.259	0.165	0.985	0.783
	F-ratio	1.16	1.51	2.41	0.00	0.08

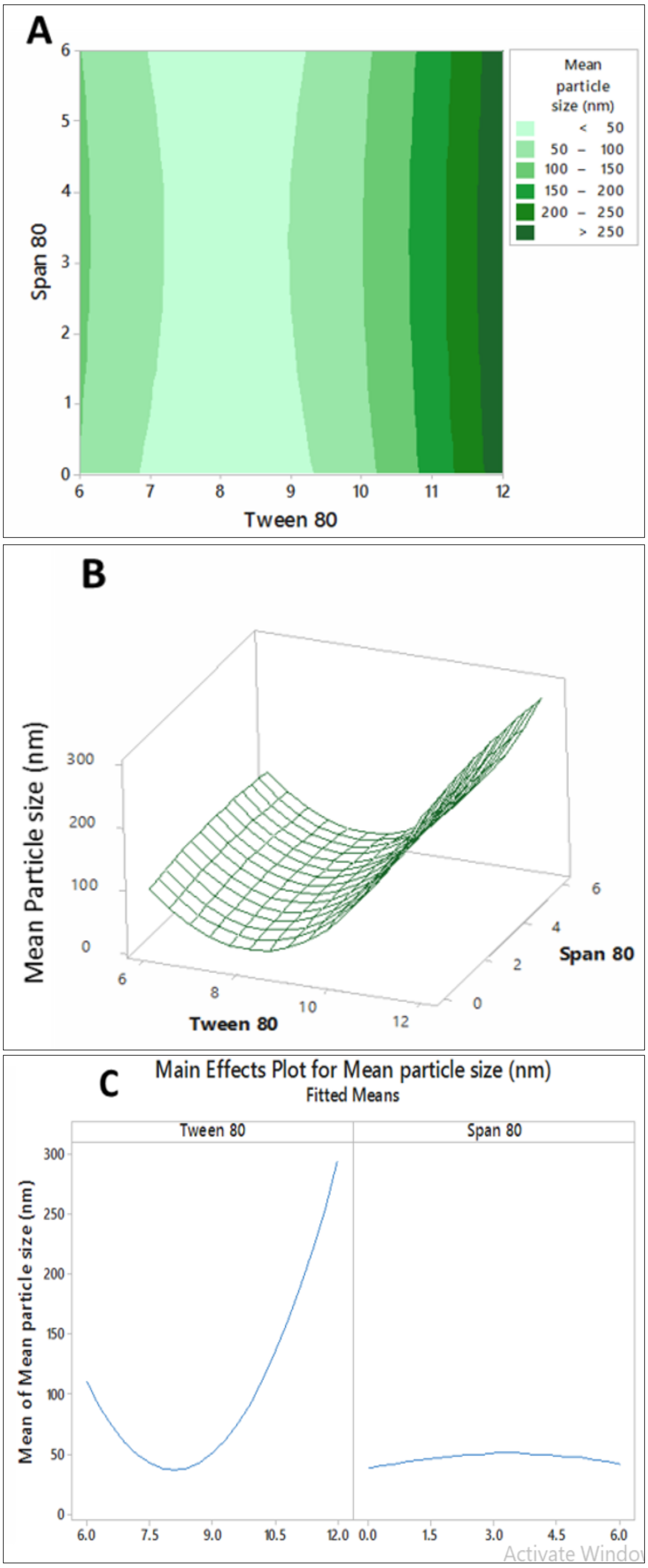

Fig. 1. Contour (**A**), Surface (**B**) and Main effects (**C**) plots for mean particle size of Germanders essential oil nanoemulsions as function of Tween 80 and Span 80 concentrations.

As mentioned in previous studies, the concentration of the emulsifiers is a crucial factor in determining the size of the obtained nanoemulsions. Generally, the particle size of these colloidal systems decreases with an increase in the concentration of the emulsifier. However, some studies have also observed an increase in the average particle size of organic nano-particles as a function of emulsifier content (24).

To prepare a stable nanoemulsion, an adequate concentration of emulsifier, particularly the hydrophilic emulsifier, is needed to cover the surface area of the newly formed nano-sized droplets. The surface area increases as the droplet size decreases. Incomplete coverage of nano-particles due to emulsifier deficiency can cause them to coalesce and increase their sizes. On the other hand, at high concentrations of emulsifier, sufficient emulsifiers are available for all newly broken nano-particles, and the emulsifier concentration is no longer a key parameter in determining the size of nano-particles. Instead, the system size distribution would be controlled by other factors like energy input. Furthermore, at high concentrations of emulsifier, the surface load of emulsifiers on droplets increases, resulting in the formation of multiple coverage layers on the nano-particles, and, subsequently, an increase in the nano-particles sizes (9, 15). Although some studies have shown that using lipophilic emulsifiers and hydrophilic ones can decrease the surface tension and act synergistically to produce smaller nano-particles, this effect was not observed in the current study. The lipophilic emulsifier did not significantly impact the mean particle size of the produced nano-particles (9, 15, 24).

As mentioned, some of the synthesized Germander essential oil nanoemulsions exhibited homogeneity with lower PDI values, while others were more heterogeneous with higher PDI values. The PDI of samples ranged from 0.1752 to 0.7824. Based on ANOVA analysis for PDI (as shown in Table 2), both linear and quadratic effects for Tween 80 and Span 80 concentrations were found to be significant in influencing PDI variations of nanoemulsions at a 95% confidence interval (p-value <0.05). The polynomial model derived for predicting PDI changes in nanoemulsions, after removing the insignificant terms, is given by Equation 3.

PDI = 1.439 - 0.2050 Tween 80 - 0.1735 Span 80 + 0.01425 Tween 80*Tween 80 + 0.01401 Span 80*Span 80 .....(Eqn. 3)

Using this equation, it is possible to predict PDI changes in the Germanders essential oil nanoemulsions based on the concentrations of Tween 80 and Span 80. This information can be valuable for optimizing the preparation of nanoemulsions to achieve the desired homogeneity and stability.

The high R^2 value of 97.11% for the model indicates that Equation 3 is highly effective in predicting the PDI values for the Germanders essential oil nanoemulsions based on the concentrations of Tween 80 and Span 80. As visualized in Fig. 2, there were optimum concentrations for both Tween 80 and Span 80 emulsifiers, which resulted in the fabrication of the most homogeneous essential oil nanoparticles with smaller PDI values. This observation suggests that by decreasing Tween 80 concentration and increasing Span 80 concentration, a significant reduction in PDI can be achieved, leading to uniform and stable nanoemulsions. The linear effect of Span 80 concentration was found to be the most significant factor influencing the PDI. Understanding the impact of both Tween 80 and Span 80 concentrations on the PDI of nanoemulsions can help

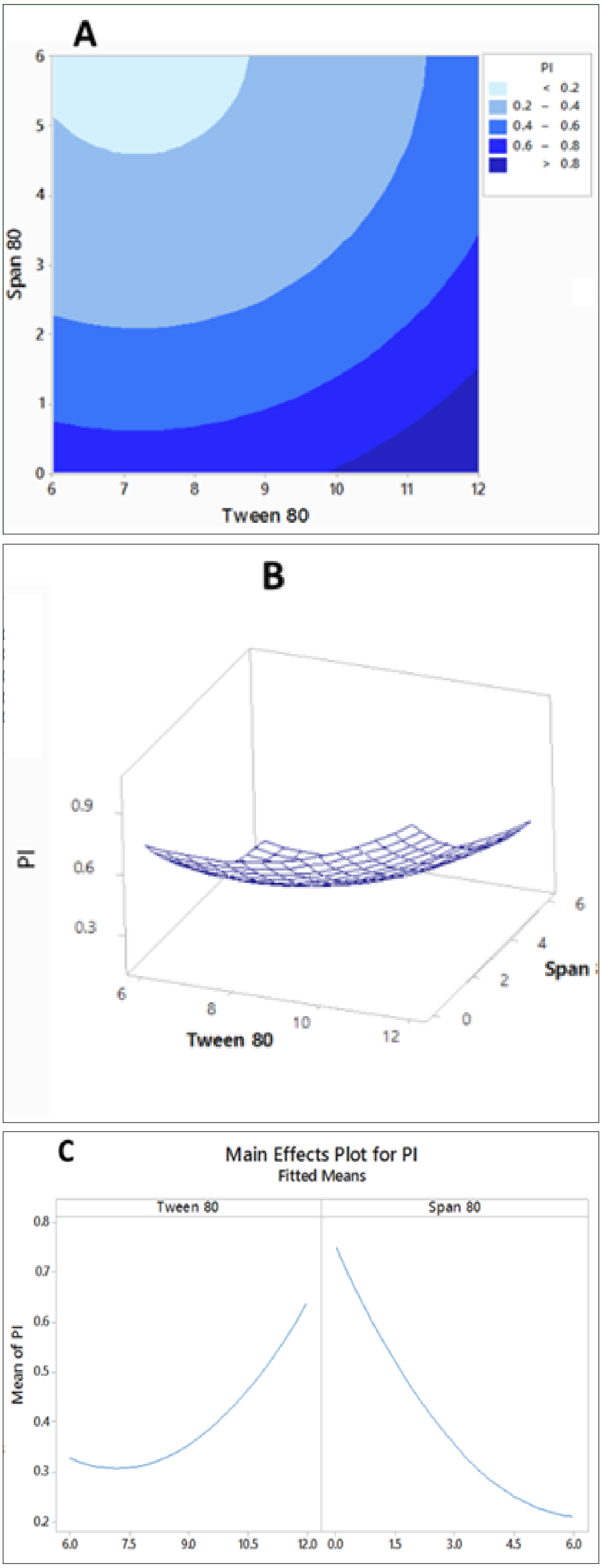

Fig. 2. Contour (**A**), Surface (**B**) and Main effects (**C**) plots for PDI of German-ders essential oil nanoemulsions as function of Tween 80 and Span 80 concentrations.

optimize the emulsifier concentrations to produce nanoemulsions with desired homogeneity and stability, which is crucial for their application in various industries such as food, pharmaceuticals, and cosmetics.

By increasing the emulsifier concentration, perfect coverage can be achieved on the nanoparticles, resulting in a more stable system. However, when the concentration of Tween 80 exceeds a certain point, excess emulsifiers can form free micelles of different sizes without an oil phase, causing considerable size heterogeneity in the system. In contrast, Span 80, as a lipophilic emulsifier, acts as a ripening retarder and balances the curvature effects by causing entropy of mixing. This prevents the diffusion of particles towards each other, ultimately hindering the attachment of nano-particles together (9, 15, 20, 25).

By incorporating the HLB parameter and total emulsifier concentration, it can be determined that both factors play a significant role in determining the mean size and size homogeneity of nanoemulsions. While an increase in both HLB and total emulsifier content can increase the PDI of the nanoemulsions, a decrease in size distribution can be observed when either the HLB is increased at lower total emulsifier concentrations or the total emulsifier concentrations are increased at lower HLB values.

An increase in the amount and hydrophilicity of the emulsifier in o/w nanoemulsions can lead to an increase in the surface area of the nano-particles. This can result in more efficient and rapid stabilization of the nano-particles, leading to smaller particle sizes due to improved coverage, wrapping, and stabilization (14). However, systems with high emulsifier content and HLB values are susceptible to the formation of free micelles of varying sizes or particles with multiple stabilizing layers, leading to a considerable increase in both PDI and particle size.

zeta-Potential and conductivity

Both hydrophilic and lipophilic emulsifiers can create a mechanical barrier that inhibits the development of surface potential, known as zeta potential. This results in the creation of repulsive forces between nanoparticles, which in turn prevents their coalescence. Nanoemulsions prepared using a mixture of Tweens and Spans as emulsifiers typically exhibit a negative zeta potential due to the adsorption of hydroxyl ions at the interface, followed by the development of hydrogen bonds between them and ethylene oxide groups of Tweens. Additionally, the essential oil components are mostly anionic and provide a negative charge to the particle surfaces (26).

As previously mentioned, a high absolute value of the zeta potential is essential for creating repulsive forces between nanoparticles (9, 15). Emulsifiers can stabilize nanoparticles through a net zeta potential greater than 30 mV is ideal for effective electrostatic repulsive stabilization of nanoparticles (11). In the prepared nanoemulsions, the zeta-potential values ranged from -2.361 to -5.042 mV, indicating that the nanoemulsions were mainly stabilized through steric repulsion and that zeta potential values did not play a vital role in their physical stability (14, 15). This is consistent with previous research that has reported lower zeta-potential values for Tween 80 stabilized carotenoid nanoemulsions (14). The lower zeta-potential values observed in the samples could be attributed to the solvent evaporation step during the preparation of the nanoemulsions, which can cause the volatilization of ionic compounds of essential oil, such as terpenoids and break up the hydrogen bonds formed between hydroxyl ions and the ethylene oxide groups of the Tweens (26).

Since no significant differences were observed in the zeta-potential values of samples (p-value>0.05), ANOVA and regression analysis was not carried out for this characteristic of Germanders essential oil nanoemulsions.

The electrical conductivity of nanoemulsions is often used to predict the volume ratio of the oil phase and the optical clarity of the system without determining its size distribution. These characteristics are effective and reliable indicators of system stability for colloidal systems with an unknown size distribution. They can also predict phase inversion destabilization in nanoemulsion systems (15).

The conductivity of prepared essential oil nanoemulsions ranged from 0.158 to 0.216 mS/cm (Table 1), placing the sample conductivities between those of pure deionized water (1.65 mS/cm) and mineral oil (0.00 µS/cm) (16). The concentration of Span 80 was found to be a more important factor than Tween 80 concentration in influencing this characteristic. As shown in Table 2, the linear effects of both Tween 20 and Span 80 and the quadratic effect of Span 80 concentrations were found to be significant in predicting changes in conductivity at a 95% confidence interval. Fig. 3 illustrates that an increase in Tween 80 concentration led to a decrease in nanoparticle conductivity. However, increasing the Span 20 content to a certain level (4.18%) caused an increase in conductivity, with further increases in Span 20 concentration resulting in a significant decrease. The reduced model for predicting the conductivity of the nanoemulsions was expressed by Equation 4.

Conductivity (mS.cm) = 0.20165 - 0.004888 Tween 80 + 0.02198 Span 80 - 0.002633 Span 80*Span 80 ....(Eqn. 4)

The obtained R^2 value for the model (R^2=96.22%) indicates that this model can predict over 96% of the variation in the conductivities of the nanoemulsions.

Additionally, the conductivity of samples decreased with an increase in the total emulsifier content of the system. It can be concluded that the presence of high surface-active molecules in these systems can reduce the transfer rate of ions by colliding with these molecules, resulting in the loss of thermal energy instead of being transferred. While conductivity is an efficient parameter for predicting the volume ratio of the oil phase and the optimal clarity of the system, the turbidity and particle size of samples were also measured in this research, indicating that conductivity alone is not a key parameter in determining the most desirable product.

Turbidity

Most of the resulting nanoemulsions had a clear or slightly turbid appearance. Based on the ANOVA analysis, the concentration of Span 80 did not have a significant effect on the turbidity of nanoemulsions. Therefore, all Span 80-related terms in the model can be removed, and the turbidity of the nanoparticles can be determined solely by the concentration of Tween 80. As shown in Fig. 4, an increase in Tween 80 concentration beyond a certain level led to an increase in the turbidity of the system.

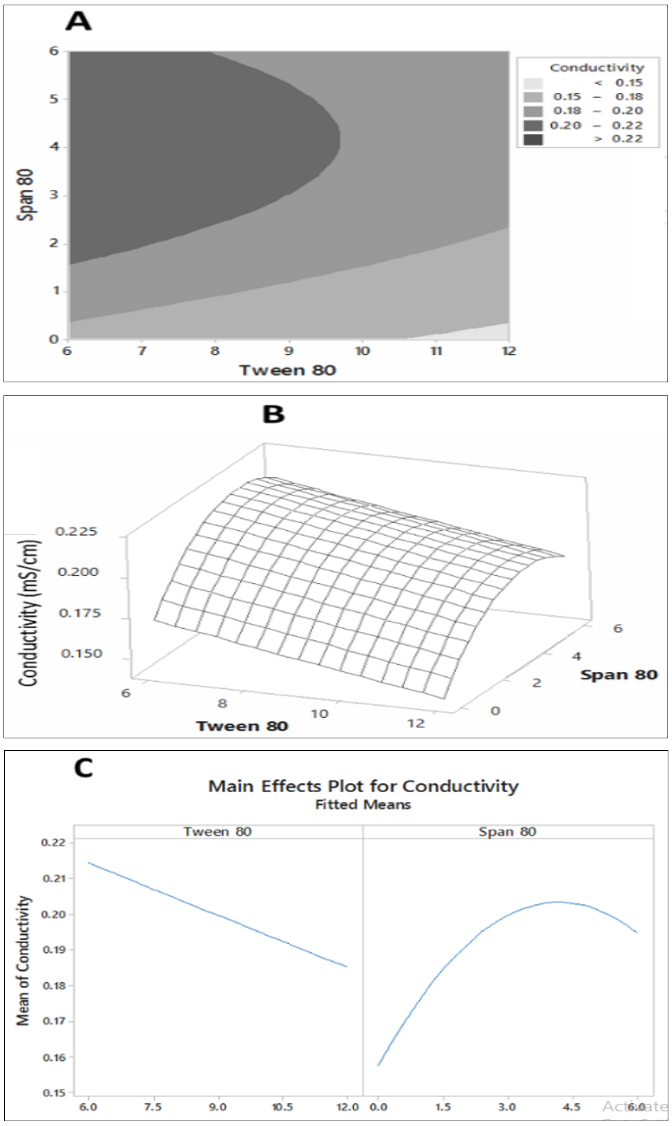

Fig. 3. Contour (**A**), Surface (**B**) and Main effects (**C**) plots for conductivity of Germanders essential oil nanoemulsions as function of Tween 80 and Span 80 concentrations.

The simplified model for predicting changes in turbidity of the nanoemulsions is represented by Equation 5, with an R^2 value of 97.00%.

Turbidity (od) = 0.1835 - 0.04590 Tween 80 + 0.003285 Tween 80*Tween 80...(Eqn. 5)

The turbidity of samples was also found to be correlated with their mean particle sizes and total emulsifier contents, as the turbidity of samples decreased with a decrease in particle size and total emulsifier contents. These findings are consistent with most previous studies (15, 27). An increase in turbidity due to an increase in emulsifier concentration is associated with excess emulsifier and free micelles in the system (27).

The single optimization approach predicted that 6.97% Tween 80 and 6% Span 80 could produce nanoemulsions with the least turbidity. The turbidity of samples remained constant after 30 days of storage at 5±1°C.

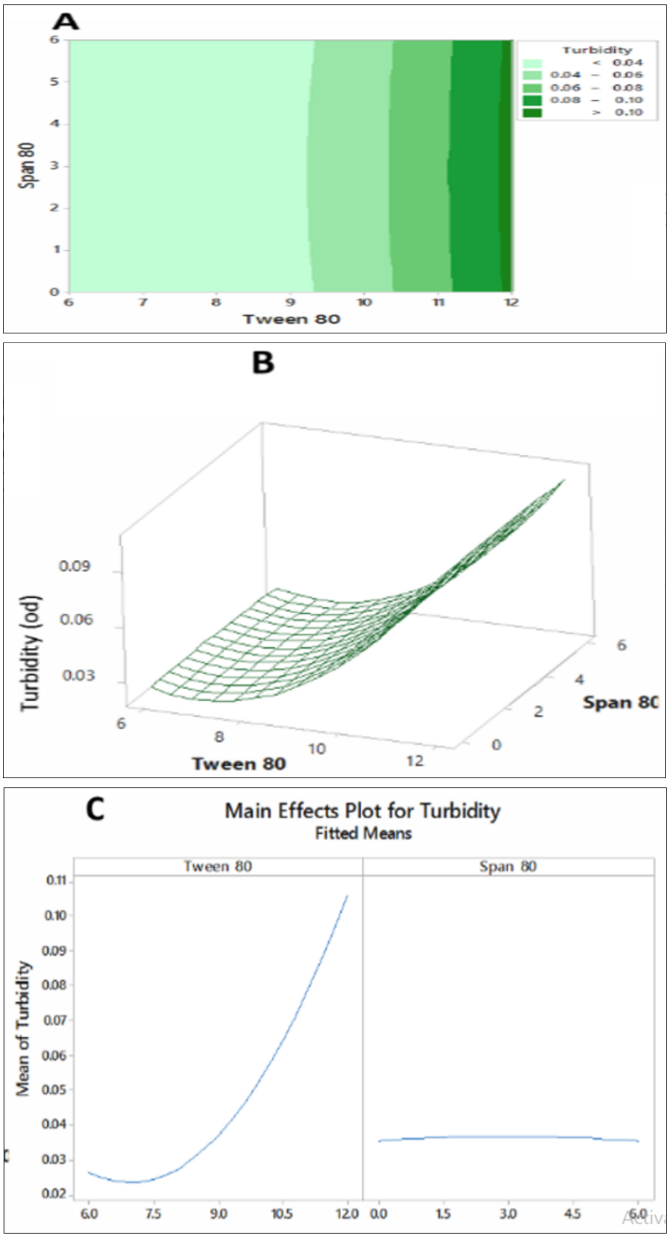

Fig. 4. Contour (**A**), Surface (**B**) and Main effects (**C**) plots for turbidity of Germanders essential oil nanoemulsions as function of Tween 80 and Span 80 concentrations.

Antioxidant and antibacterial activities

According to the "polar paradox theory", lipid-based antioxidants are more effective in o/w dispersion systems than bulk oils. This can be attributed to their higher affinity for the o/w interface, where they create a protective layer around the particles. However, some exceptions have been reported in this regard. While the effects of most process and formulation parameters on the characteristics of nanoemulsions have been widely studied, there are limited reports on the antioxidant activities of nanoemulsions containing functional lipid bio-actives, particularly essential oils with mixtures of chemical compounds. It has been found that the antioxidant activities of these samples depend greatly on the chemical structure and content of their oil phase components, and their size (28).

The antioxidant activities of the synthesized nanoparticles were found to be inversely correlated to their sizes (R^2 = 91.3%), which is consistent with most previous research. Since pure Tween 80 and Span 80 showed no DPPH radical scavenging activity only the essential oil components were likely responsible for the antioxidant activities of samples. The H-atoms of the directly bonded hydroxyl group to the aromatic ring in phenolic and other organic composites of Germanders essential oil can trap peroxyl radicals and prevent other compounds from oxidation (17). The lower DPPH radical scavenging activities of Germanders essential oil in macro-sized form, even in comparable concentration to the nano-sized form (which was equal to 43.5±1.9%), provides good evidence for enhancement of the antioxidant activities of essential oils through size reduction into the nano ranges and their incorporation into nanoemulsion systems (3, 7).

The antioxidant activities of nanoemulsions can serve as a good indicator of the retention of active compounds from Germanders essential oil during the size reduction process. The analysis of the sample capacity for quenching DPPH-free radicals ranged from 57.52% to 89.56% (Table 1). The impact of stabilizer doses on the antioxidant activity of Germanders essential oil nanoemulsions is illustrated in Fig. 5. As shown in this figure, an increase in both Tween 80 and Span 80 concentrations mostly resulted in a decrease in the samples' antioxidant activities. The suggested polynomial model in a 90% confidence interval was expressed by Equation 6, with an R^2 value of 97.86%.

DPPH Scavenging (%) = 13.28 + 20.71 Tween 80 - 0.714 Span 80 - 1.3948 Tween 80*Tween 80.................. (Eqn. 6)

At high concentrations of emulsifiers, the essential oil components of the nanoemulsions may be difficult to release from the possible multi-layer stabilizing barrier that is created. When they are released, they may become trapped in free micelles. Consequently, their presence and scavenging activities could be restricted in the aquatic environment, leading to a decrease in the antioxidant activity of the samples at higher emulsifier concentrations.

The single optimization approach aimed at producing Germanders essential oil nanoemulsions with the highest antioxidant activity suggested that the most desired samples could be obtained using 7.4% Tween 80 and 0% Span 2 to synthesize the nanoemulsions.

The antibacterial activities of the obtained Germanders nano-particles were evaluated against *Staphylococcus aureus* and *Salmonella enterica* as representative Gram-positive and Gram-negative bacteria, respectively. All samples exhibited similar antibacterial activities against *S. aureus* (10.46±0.366 mm) and *S. enterica* (12.53±0.338 mm). Therefore, in the present study, the concentrations of emulsifiers and the size of the obtained essential oil did not play an important role in the bactericidal activities of the nanoparticles. The antibacterial activities of macro-sized nano-particles in a similar concentration to nano-sized ones were 5.25± 0.555 and 6.10±0.255 nm against *S. aureus* and *S. enterica*, respectively. Thus, the size reduction of Germanders essential oil into the nano-ranges could increase its antibacterial activities by approximately 70% against Gram-positive *S. aureus* and 170% against Gram-negative *S. enterica.* It can be concluded that the incorporation of Germanders essential oil into nanoemulsion systems could enhance their bactericidal activities, particularly against Gram-negative bacteria.

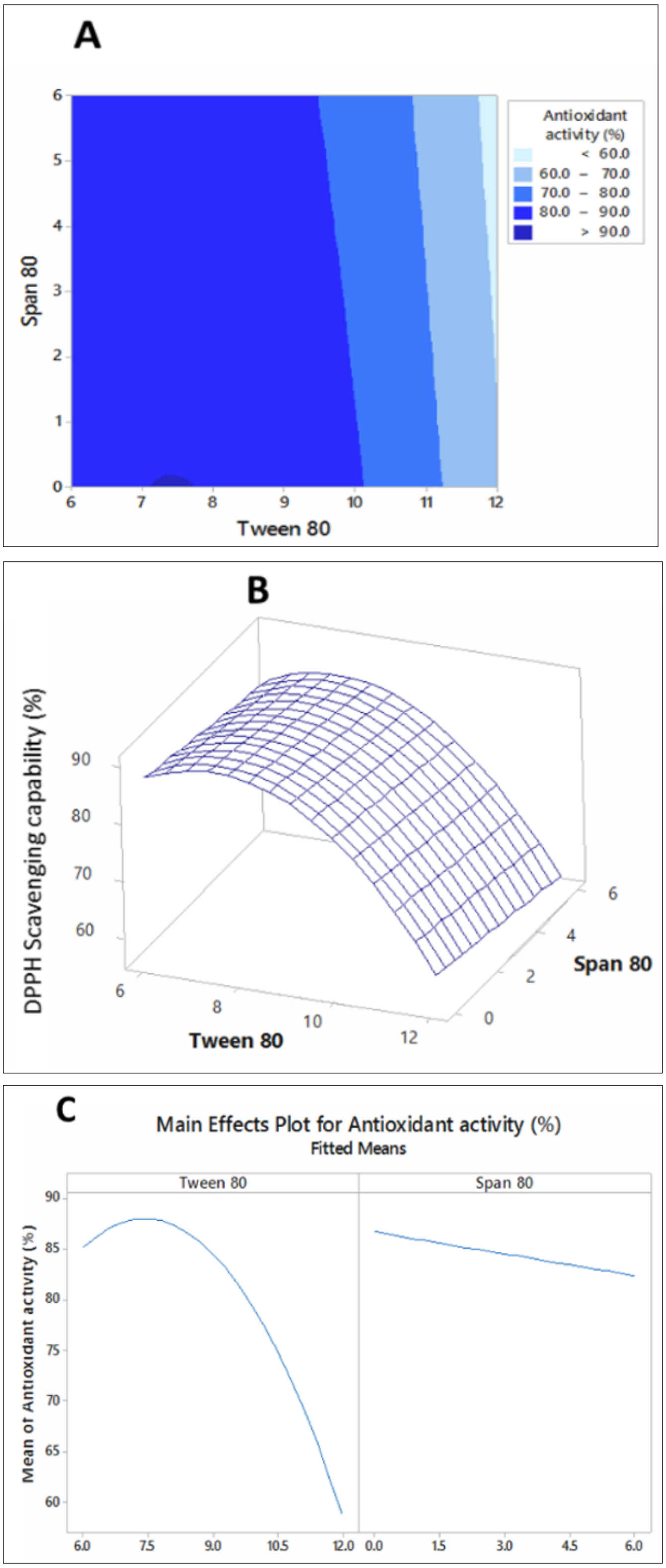

Fig. 5. Contour (**A**), Surface (**B**) and Main effects (**C**) plots for antioxidant (DPPH radical scavenging) activity of Germanders essential oil nanoemulsions as function of Tween 80 and Span 80 concentrations.

The chemical components of essential oils can attach to the bacterial cell membrane and disrupt its normal action, leading to the inhibition or expansion of its permeability and ultimately killing the cells by limiting the input of required components or releasing cell content. These compounds can also bond to amino acids, proteins, nucleic acids, and other cell components, disrupting their regular functionalities in metabolic reactions. Regarding Germanders essential oil, its chemical components seem to disrupt the external layer of Gram-negative strains, inactive endotoxins, and increase cell penetrability. They may also decrease the potassium particles of these cells by substituting their hydroxyl gathering, and reduce the level of intracellular ATP during the expansion of extracellular ATP. Some segments of Germanders essential oil, such as Pulegone, can also block the development of certain enzymes, like amylase, particularly in Gram-negative bacteria (17, 29). The ANOVA and regression analysis were not performed on the bactericidal activities of samples due to the observed similar antibacterial actions for all samples.

Optimization

The optimal Germanders nanoemulsions were achieved by applying numerical or graphical statistical optimization processes to determine the best emulsifier concentrations. The white-colored region in Fig. 6 shows the ideal emulsifier concentration for preparing Germanders nanoparticles. The best concentrations of emulsifiers were found to be between 7-8% Tween 80 and 3.6-6% Span 80. Moreover, according to numerical multiple optimization processes, 7.3% Tween 80 and 5.6% Span 80 would produce the most desired Germanders nanoemulsions. These nanoemulsions have the smallest mean particle size (41.5 nm), PDI (0.1739), turbidity (0.00227 od), and the highest antioxidant activities (86.11%), with a composite desirability of 0.9644. Notably, the numerical optimization result falls within the optimum region of the graphical optimization process.

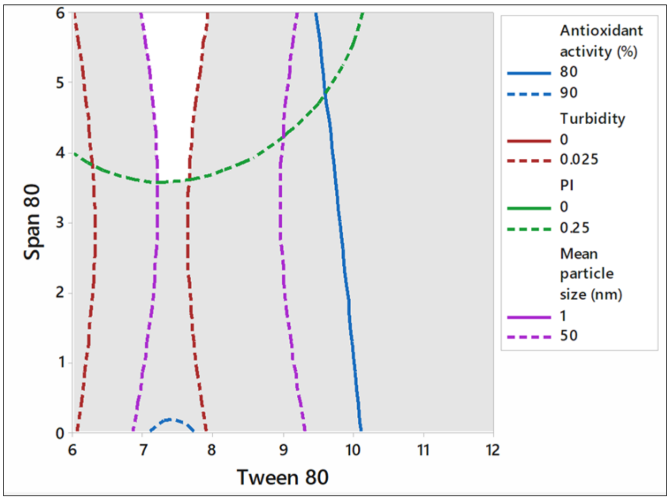

Fig. 6. The overlaid contour plot of the optimization process in order to optimize the Tween 80 and Span 80 concentrations, leading to the most desired Germanders nanoemulsions.

Validation of models

The adequacy of the proposed models for predicting each studied characteristic was confirmed through several measures. First, by comparing a plot of the experimental values to those predicted by the final reduced models, linear plots with intercepts of zero, a slope of 1, and high

R^2 values (>0.90) demonstrated the model's adequacy. Additionally, the acquired p-values from the comparison t-test analysis between the anticipated and experimental values of the responses supported their general proximity (1.00 for all responses). Furthermore, to validate the models, three replications were prepared using the optimum concentrations of emulsifiers, 7.3% Tween 80 and 5.6% Span 80. The measured characteristics were as follows: mean particle size: 44.1 ± 2.45 nm, PDI: 0.1699 ± 0.020, turbidity: 0.0020 ± 0.0005 od, conductivity: 0.217 ± 0.011 mS/cm, net zeta-potential: 4.18±0.044 mV and DPPH radical scavenging: 86.61 ± 1.08% and *Staphylococcus aureus* growth inhibition: 10.5 mm and *Salmonella enterica* growth inhibition: 12.7 mm. The comparison of Tukey test results revealed no significant difference between the measured characteristics and those predicted by the models for Germanders nanoemulsions prepared using optimum concentrations of emulsifiers. Thus, the correctness of the models was re-confirmed, indicating that these models reliable for predicting the properties of Germanders nanoemulsions within the studied range of variables.

Stability studies

The physicochemical stability of optimal Germanders essential oil nanoemulsions was evaluated by analyzing the average particle size, PDI, turbidity, antioxidant, and antimicrobial activities after 30 days of storage at 5°C. The results indicate that the turbidity and optimal average size of Germanders nanoemulsions remained fairly constant during storage (turbidity and mean particle size of stored samples at 5°C after 30 days were 0.0021±0.0004 and 75± 3.10 nm, respectively). However, the PDI of samples increased slightly during storage (after 30 days at 5°C, the PDI of a fresh sample was 0.1699±0.020 and that of a sample held at 5°C was 0.1758±0.005, respectively). The anti-oxidant and antibacterial properties of the optimal Germanders essential oil nanoemulsions decreased after storage at 5°C. The antioxidant activity and *Staphylococcus aureus* and *Salmonella enterica* growth inhibition zone of samples after storage were 73.80±0.8 % and 8.5±1 and 9.9±0.6 mm, respectively. Based on these findings, it can be concluded that the physical and chemical stabilities of the samples were acceptable but limited, indicating that the nanoemulsions may be best used or consumed shortly after preparation to ensure optimal effectiveness.

Morphology

The morphology of optimum Germanders nanoemulsions was examined using transmission electron microscopy (TEM) images (Fig. 7). The TEM images revealed that the prepared nano-sized essential oils droplets were spherical with a size of approximately less than 50 nm. These findings are consistent with the results obtained from the zeta-sizer, which were based on dynamic light scattering mechanisms. This agreement between the two methods further validates the size and morphology of the prepared Germanders essential oil nanoemulsions, indicating that the optimization process successfully produced the desired nanoemulsion characteristics.

Chemical compositions of Germanders essential oil

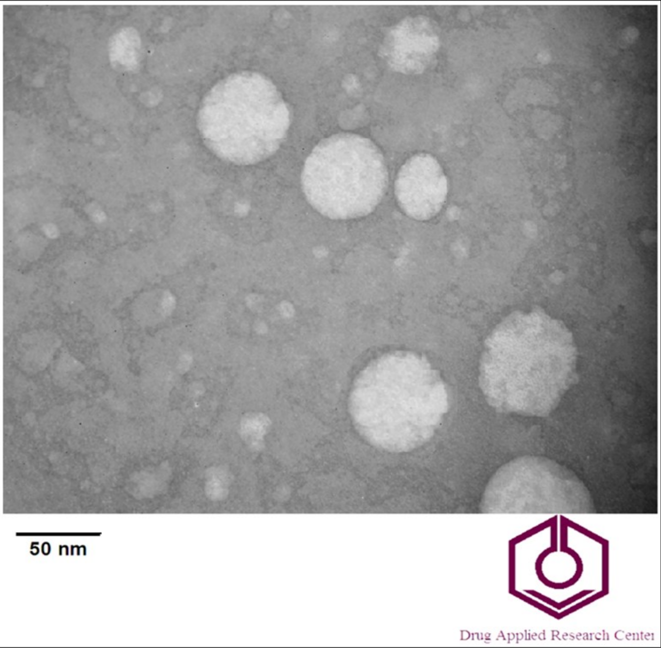

Fig. 7. The TEM image of Germanders nanoemulsions (prepared at optimum emulsifiers' concentrations, 7.3% Tween 80 and 5.6% Span 80).

Table 3 presents the components of Germanders essential oil identified using gas chromatography-mass spectrometry (GC-MS). According to Table 3, approximately 50 constituents were detected. The primary constituents of Germanders essential oil included: γ-muurolene (8.55%), 1,8 cineole (7.6%), terpineol (6.3%), sabinene (5.9%), and pinocarveol (5.2%).

Many of these identified compounds possess a variety of pharmacological effects, such as antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, anticancer, hypoglycemic, hypolipidemic, and hepatoprotective properties. For instance, γ-muurolene has been shown to exhibit strong cytotoxicity against various tumor cell lines (30). 1,8-Cineole is a cyclic ether mono-terpenoid compound with a pleasant, spicy taste and aroma, and previous research has reported its anti-inflammatory properties (31). Terpineol and pinocarveol belong to the oxygenated monoterpenes group and are known for their antioxidant and antibacterial activities. Additionally, their pleasant smell makes them suitable candidates for use in fragrances, cosmetics, and flavorings (31). Sabinene is a bicyclic monoterpene with demonstrated antioxidant, anti-inflammatory, anti-fungal, and antibacterial properties (32). The presence of these bioactive compounds in Germanders essential oil highlights its potential for various applications in the pharmaceutical, cosmetic, and food industries.

Caryophyllene (3.5%), α-humulene (3.3%), δ-cadinene (3.1%), turpentine (2.4%), germacrene D (2.3%), and α-pinene (2.2%) are some other components of Germanders essential oil. Previous studies on Jordanian Germanders have identified caryophyllene, germacrene D, and sabinene as the main components of the essential oil, albeit in different concentrations (33) . The primary components of Saudi Arabia-grown Germanders essential oil were also γ-muurolene, cadinol, cadinene, pinene,

Table 3. Chemical composition of the Germanders essential oil.

Sl. No	Name of chemical component	Percentage of chemical component (%)	Retention time (min)
1	2,5-diethyltetrahydrofuran	0.5	6.03
2	α-PDInene	2.2	7.04
3	sabinene	5.9	8.19
4	β-PDInene	1.8	8.35
5	turpentin	2.4	8.48
6	myrcene	0.8	8.65
7	3-octanol	1.2	8.94
8	phellandrene	0.1	9.02
9	1,8-cineole	7.6	9.97
10	α-ocimene	0.5	10.04
11	α-thujone	1.6	10.43
12	linalool	1.1	10.98
13	butanoic acid	0.24	11.96
14	PDInocarveol	5.2	12.03
15	3-Octyl acetate	0.28	12.51
16	terPDIneol	6.3	12.66
17	mirtenal	0.7	13.55
18	thymol	0.2	14.05
19	Estragole	0.9	14.77
20	dibutyl ester	0.1	16.44
21	p-mentha-1,4 dien-7-ol	0.5	17.06
22	cis-PDIperitone oxide	1.32	17.18
23	pulegone	0.17	17.23
24	isopulegyl acetate	0.19	17.31
25	ethanone 1-(2,5-	0.07	17.50
26	Menthyl Acetate	1.57	17.70
27	p-cymene	1.1	17.97
28	limonene	1.3	18.02
29	PDIperitone	0.06	18.50
30	cubebene	0.3	18.82
31	α-gurjunene	0.2	19.05
32	PDIperitenone oxide	0.9	19.51
33	β-bourbonene	0.1	19.92
34	β-elemene	0.1	20.06
35	β-caryophyllene	3.5	20.88
36	γ-muurolene	8.6	21.24
37	nerylacetone	0.6	21.40
38	α-humulene	3.3	21.53
39	allo-aromadendrene	0.9	21.91
40	cuparene	0.5	21.99
41	δ-cadinene	3.1	22.24
42	germacrene D	2.3	22.35
43	germacrene B	0.4	22.69
44	spathulenol	0.3	24.62
45	caryophyllene oxide	0.4	24.75
46	dillaPDIole	0. 9	25.51
47	α-cadinol	2.2	25.76
48	isospathulenol	0.1	25.96
49	4-methyl-3-oxopentanal	0.1	26.01
50	iso-cedranol	1.3	26.93

β-gurjurene, α-limonene, α-pinene, and sabinene, but with different contents compared to the present study (34). The varying proportions of main components in Germanders essential oil from different regions can be attributed to differences in genetic factors, vegetative phases, and environmental conditions such as climate and soil structure. These variations can influence the overall composition and the potential applications and benefits of the essential oil extracted from Germanders grown in different locations.

Conclusion

In summary, nanoemulsions of Germanders essential oil were successfully prepared using a solvent-displacement technique with varying concentrations of Tween 80 and Span 80 as emulsifiers. The concentrations of these emulsifiers were determined to have significant effects on the physical and chemical properties of the resulting essential oil nanoemulsions. Multiple regression analysis was employed to develop models predicting the average particle size, PDI, zeta-potential, conductivity, turbidity, and antioxidant properties of nano-sized Germanders essential oils as a function of emulsifier concentrations. The optimal formulation, which exhibited the smallest particle size, PDI, conductivity, turbidity and the highest net zeta-potential and antioxidant activity, was achieved using 4.8% Tween 80 and 7.8% Span 80. Compared to their micro-sized counterparts, the prepared Germanders essential oil nanoemulsions demonstrated enhanced antioxidant activities and improved antibacterial properties against Gram-positive or Gram-negative bacterial strains. These nano-sized Germanders essential oil emulsions, with their high physical stability and potent bioactivities, could potentially be utilized as natural preservatives in various food and beverage products or as biocompatible air disinfectants in hospitals and patient rooms. This innovative approach to harnessing the benefits of Germanders essential oil in nanoemulsion form offers promising opportunities for its application in various industries, including food preservation, cosmetics, and healthcare.

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Authors contributions

All Authors have made substantial contributions to conception and design, acquisition analysis and interpretation of data, and drafting the manuscript. All authors also agreed to be responsible for all aspects of the work in certifying that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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