

## Research Article

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# New approach in process intensification based on subcritical water, as green solvent, in propolis oil in water nanoemulsion preparation

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**Abstract:** Subcritical water was used to provide propolis oil in water (O/W) nanoemulsions. To monitor and detect the main bioactive compounds of the prepared propolis extract, gas chromatography demonstrated that there were 47 bioactive materials in the propolis extract, among which pinostrobin chalcone and pinocembrin were the two key components. Effectiveness of two processing parameters such as the amount of saponin (0.5–2.0 g) and propolis extract (0.1–0.6 g), on particle size, polydispersity index (PDI), zeta potential, and antioxidant activity of the provided nanoemulsions, was evaluated. Results demonstrated that more desirable propolis O/W nanoemulsion, with minimum particle size (144.06 nm) and PDI (0.286), and maximum zeta potential (−21.71 mV) and antioxidant activity (90.86%) were made using 0.50 g of saponin and 0.53 g of propolis extract. Further analysis revealed that the prepared nanoemulsion based on optimum processing conditions had spherical shaped propolis nanodroplets in the colloidal solution with turbidity and maximum broad absorption peak of 0.08 a.u. and 292 nm, respectively. The prepared nanoemulsion had high antibacterial

activity against both selected bacteria strains namely, *Staphylococcus aureus* and *Escherichia coli*.

**Keywords:** oil in water nanoemulsion, propolis extract, process intensification, saponin, subcritical water

## 1 Introduction

Natural products, with distinctive biological and pharmaceutical attributes, have gained more attentions by numerous industries such as food, medicine, and agriculture, these days. Among these valuable natural compounds, propolis has been the subject of interest in traditional medicine because of its application in treating wounds and burns, sore throat, and stomach ulcer [1,2]. Propolis is also known as bee glue, which is a sticky resinous compound and provided by bees (*Apis mellifera*) from different parts of plants such as flower, pollen, buds, branch, and exudates of tree [3–5]. It mainly contains plant resin (50%), bees wax (30%), essential oils and aromatics (10%), pollen (5%), and several valuable bioactive components such as flavonoids, terpenoids, steroids, ketones, phenolic acids and aldehydes, minerals, and vitamins [4,6–8]. Therefore, in modern pharmaceuticals and medicine areas, it has been widely used as antibacterial and antiviral, immunomodulating, anti-inflammatory, anti-tumor, and anti-obesity agents [3–6,8,9].

Because of non-polar nature of propolis, its applications in aqueous food and drug formulations have been limited. However, by development of nanotechnology, it is possible to reduce droplet and droplet size of hydrophobic bioactive components to less than 1,000 nm and increase the ratio of their surface to volume which that improves their solubility in watery-based solutions [10,11]. Nanoemulsions, as isotropic colloidal matrices, are composed of three main components, namely liquid and organic phases, and an amphiphilic interfacial stabilizing agent, which is known as emulsifier [10,12–15]. The structure of oil in water (O/W) nanoemulsions is similar to

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nanocapsule matrices which in those, lipophilic compound, as core, is covered by an amphiphilic layer made by surface active molecules [16]. In fact, O/W nanoemulsions may be used as carrier systems of non-polar materials such as nutraceuticals, flavor and aroma components, antioxidants, and antimicrobial agents in aqueous media [17].

Several methods have been used to prepare O/W nanoemulsions, which are classified into two major groups namely high and low energy consuming methods [10,16,18–20]. Subcritical water (SW) is hot and pressurized water with temperature and pressure of higher than 100°C and 1 atm, respectively, which water preserves its liquid state. Polarity of SW varies to that of the methanol, ethanol, and acetone, which help to solubilize non-polar compounds in itself [10,21–23]. Using SW in production of O/W nanoemulsions, as a novel approach of using green solvent to intensify of the process of nanoemulsion preparation, has several advantages as compared to other common nanoemulsion preparation methods, such as need for minimum amounts of emulsifier and no need for the removal of the solvent, which make the process cost-effective and eco-friendly [15,22,24]. Several studies have been completed to produce O/W nanoemulsions using SW. Ahmadi and Jafarizadeh-Malmiri produced thyme O/W nanoemulsions using SW at 120°C and 1.5 atm, for 2 h, and using saponin and xanthan gum, as emulsifiers [10,25]. Sayyar and Jafarizadeh-Malmiri prepared curcumin O/W nanodispersions using SW and mentioned operation conditions [21–23]. They successfully decreased amount of emulsifiers such as saponin, xanthan gum, and Tween 80 and 20, which were used in the production of O/W nanoemulsions.

Core purposes of this research were to: (i) prepare propolis extract and evaluate its characteristics, (ii) produce propolis O/W nanoemulsions using SW and saponin as solvent and emulsifier, respectively, (iii) evaluate the effectiveness of saponin and propolis extract amounts on some physico-chemical attributes of the provided nanoemulsions, (iv) optimize the process parameters in propolis O/W nanoemulsion preparation, using response surface methodology (RSM), and (v) study the characteristics of the resulted nanoemulsions using attained optimum processing conditions.

## 2 Materials and methods

### 2.1 Materials

Raw propolis was purchased from a herbal market in Tabriz (Iran). Saponin and 2,2-diphenyl-2-picrylhydrazyl

(DPPH) were provided from Sigma-Aldrich Company (St. Louis, MO, USA). Bacteria strains of *Staphylococcus aureus* and *Escherichia coli* were obtained from PTCC (microbial Persian Type Culture Collection, Tehran, Iran) with ID numbers of 1,112 and 1,270, respectively. Plate count agar (PCA) was provided from Oxoid (Oxoid Ltd., Hampshire, England). Ethanol 96% was used to prepare propolis extraction and double-distilled water was used for nanoemulsion preparation.

### 2.2 Methods

#### 2.2.1 Providing propolis ethanolic extract

To prepare the propolis extract, raw propolis was decontaminated from dusts and other bee residuals manually. After that it was converted to thin sheets manually and kept in a freezer (–20°C) over night. Frozen propolis sheets were then powdered using a domestic grinder (Nima-NM-8300-Silver, Tokyo, Japan). Ethanolic extract of propolis was provided using modified method presented by Paviani et al. [26]. In this technique, 3 g of propolis powder was added to 10 mL of 96% ethanol and mixed using a hotplate magnetic stirrer (Termix-200, Tehran, Iran), adjusted at 200 rpm and room temperature (30°C) for 24 h. After that, sample was filtered using filter paper, and the filtrate was stored in a freezer at –10°C overnight, to remove its wax by its centrifugation using a laboratory centrifuge (Universal 320 R, Hettich Zentrifugen, Germany) device set at 4,500 rpm and 30°C, for 10 min, and then the supernatant was separated. Finally, the sample was evaporated using a vacuum rotary evaporator (TAT-Rdig Co., Tehran, Iran) adjusted to a temperature of 45°C to remove ethanol and prepare the propolis extract. The extract was then stored at 4°C for further use.

#### 2.2.2 Preparation of propolis O/W nanoemulsions

According to the design of experiment (Table 1), 0.5–2 g saponin was dissolved in 45 mL distilled water and those were mixed together with a laboratory heater and stirrer, set at 60°C and 200 rpm, for 15 min. Thereafter, provided propolis extract with different amounts of 0.1–0.6 g was mixed with the solutions using heater and stirrer for another 5 min. At the end of process, the provided solutions were added into 100 mL hydrothermal autoclave and put in a laboratory oven (Behdad Medical Production

**Table 1:** CCD and values of response variables (predicted and experimental) for preparation of propolis O/W nanoemulsions

Runs	Amount of propolis extract (g)	Amount of saponin (g)	Particle size (nm)		PDI		Zeta potential (mV)		Antioxidant (%)	
			Exp <sup>a</sup>	Pre <sup>b</sup>	Exp <sup>a</sup>	Pre <sup>b</sup>	Exp <sup>a</sup>	Pre <sup>b</sup>	Exp <sup>a</sup>	Pre <sup>b</sup>
1	0.350	1.250	633.0	633.3	0.694	0.694	-17.8	-17.8	86.1	86.1
2	0.350	1.250	633.0	633.3	0.694	0.694	-17.8	-17.8	86.1	86.1
3	0.600	1.250	334.0	312.4	0.548	0.513	-17.8	-17.6	83.6	83.0
4	0.350	2.000	382.0	382.0	*	0.874	-14.0	-13.9	85.5	84.3
5	0.350	1.250	614.0	633.3	0.713	0.694	-18.5	-17.8	87.2	86.1
6	0.173	1.780	146.0	115.4	0.787	0.770	-15.4	-15.3	86.0	86.6
7	0.526	1.780	492.0	513.6	0.769	0.790	-15.2	-15.3	79.8	80.8
8	0.100	1.250	207.0	258.8	0.691	0.710	-19.4	-19.4	*	85.4
9	0.350	1.250	528.0	633.3	0.675	0.694	-17.1	-17.8	87.4	86.1
10	0.350	0.500	537.0	567.9	0.532	0.516	-23.0	-22.9	89.1	89.6
11	0.350	1.250	759.0	633.3	*	0.694	*	-17.8	83.7	86.1
12	0.526	0.719	*	284.8	0.345	0.377	-20.2	-20.3	88.9	88.7
13	0.173	0.719	659.0	607.2	0.683	0.676	*	-22.9	86.8	86.2

\*Out of range.

<sup>a</sup> Experimental values of studied responses. <sup>b</sup> Predicted values of studied responses.

Co., SP88, Tehran, Iran). The processing conditions in the sealed Teflone, placed in autoclave, were temperature and pressure of 120°C and 1.5 atm, for 120 min [10].

## 2.3 Analysis

### 2.3.1 Propolis extract

Bioactive components of the provided propolis extract were detected by gas chromatograph (GC-MS, Agilent 6890, Santa Clara, CA, USA) coupled with a 5989A mass spectrometer and a HP-5 MS capillary [25]. pH of the extract was evaluated using a laboratory pH meter and its brix value was measured using a refractometer.

### 2.3.2 Propolis nanoemulsions

To evaluate the nanodroplet size of propolis and its distribution in the provided nanoemulsion, and polydispersity index (PDI) and zeta potential values of the provided nanoemulsions, a particle size analyzer (Malvern instruments, Zetasizer Nano ZS, Worcestershire, UK) based on dynamic light scattering (DLS) was used. Furthermore, this instrument has potential application to plot zeta potential distribution of the provided samples. All characteristics were repeated thrice and the mean values of data were reported. It notes that PDI is an index that shows the homogeneity and uniformity of the formed propolis nanodroplets in the prepared nanoemulsions,

and its value has changed between 0 and 1 [27]. Appearance of the formed nanoemulsions, based on their turbidity, was evaluated by a UV-Visible spectrophotometer set at 625 nm (wavelength). Fabrication of the propolis nanoemulsions was qualitative verified, based on their surface plasmon resonance (SPR) characteristic, which that can be observed as highlighted peak and detected by a UV-Vis spectrophotometer adjusted at wave length between 200 and 420 nm [22]. To evaluate the microstructure attributes of the formed propolis nanodroplets in the samples, such as shape and size, a transmission electron microscopy (TEM, CM120, Philips Co., Amsterdam, The Netherlands) was used.

Antioxidant activity of the resulted nanoemulsions was calculated using the method described by Ahmadi and Jafarizadeh-Malmiri, based on free radical scavenging activity of DPPH, and its value was reported by percentage of scavenging ability (Eq. 1) [25]:

$$I\% = (A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}} \times 100, \quad (1)$$

where  $I\%$  is the inhibition percent and  $A$  is the recorded absorbance of control (DPPH) and sample (nanoemulsions) at 517 nm (wavelength) which were obtained using spectrophotometer (250–800 nm, Perkin Elmer's Co., Rodgau, Germany).

Bactericidal effects of the propolis extract, saponin, and provided propolis nanoemulsions were monitored according to agar diffusion method [10,21]. In this method, 0.1 mL of prepared suspension of *S. aureus* and *E. coli* having  $1.5 \times 10^8$  colony forming unit (cfu) was spread on the set PCA surface, and three holes with a diameter of 5 mm were created in the plates. After that, 10  $\mu$ L of the samples were added into the holes and the plates were

then incubated at 37°C for 24 h. Antibacterial activity of the samples could be manifested in the formed clear zone around the holes.

### 2.3.3 Experimental design and data analysis

Minitab statistical software (V: 19.2020.1, Minitab Inc., Pennsylvania State, PA, USA) was used for experimental design, model, and optimization of the processing conditions for the preparation of nanoemulsions and analysis of variance. Based on this software, central composite design (CCD) with 13 randomized experiments (Table 1) and RSM were used and processing factors were the amounts of propolis extract ( $X_1$ , 0.1–0.6 g) and saponin ( $X_2$ , 0.5–2 g), and responses were particle size ( $Y_1$ , nm), PDI ( $Y_2$ ), zeta potential ( $Y_3$ , mV), and antioxidant activity ( $Y_4$ , %) of the provided nanoemulsions. To correlate the independent parameters with the responses, second-order polynomial model (Eq. 2) was used.

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j. \quad (2)$$

In this equation, the linear ( $X_1$ ,  $X_2$ ), quadratic ( $X_1^2$ ,  $X_2^2$ ), and interaction ( $X_1$ ,  $X_2$ ) terms of independent factors had coefficients of linear ( $\beta_i$ ), quadratic ( $\beta_{ii}$ ), and interaction ( $\beta_{ij}$ ) and  $\beta_0$  was constant [10]. As compared to other statistical methods, RSM has potential applications to show the interaction effect of the independent variables on the responses. Furthermore, it can be model the process and predicted the values of responses, as function of independent variables, with minimum experimental runs [22]. Coefficient of determination ( $R^2$ ) and lack-of-fit based on  $p$ -value were used to evaluate desirability and adequacy of the models [24]. Furthermore, small  $p$ -value ( $p < 0.05$ ) was selected to evaluate the significance of the model terms [25]. Contour and surface plots were stabilized to well imagine the effects of processing parameters on the responses and determine the optimum amounts of processing conditions for each response [22]. Numerical and graphical optimizations, based on overlaid contour plots, were carried out to find optimum amounts of saponin and propolis extract in the preparation of nanoemulsion, which had small nanodroplet size and PDI values, and high zeta potential and antioxidant activity values.

## 3 Results and discussion

### 3.1 Propolis extract attributes

According to the attained results, turbidity, pH, and brix of the extracted propolis had values of 1.091% a.u.,

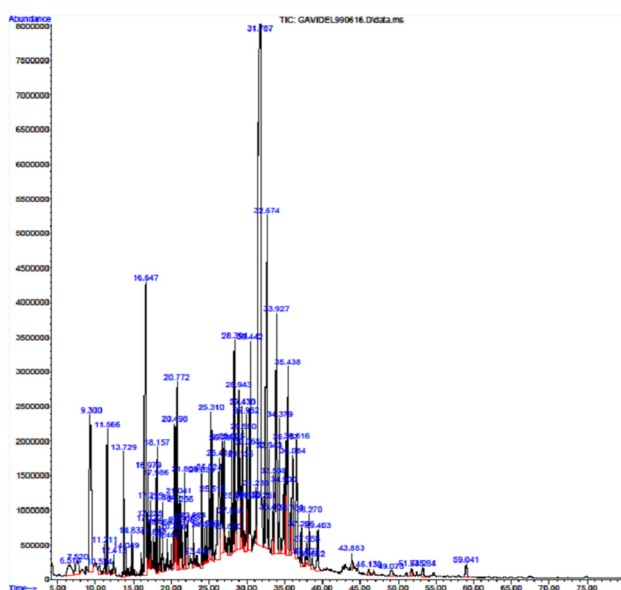


Figure 1: GC-MS chromatogram of the propolis extract.

5.9°Bx and 18.5°Bx, correspondingly. GC-MS chromatogram of the provided extract shows 82 highlighted peaks within 80 min of retention time, of which 47 compounds could be detected (Figure 1). The main compounds of propolis extract were pinostrobin chalcone (22.90%), pinocembrin (6.14%), 2,1,3-benzothiadiazole (5.76%), tectochrysin (4.83%), phenethyl alcohol (4.25%), and oleic acid (3.10%), where those were centered at retention times of 31.78, 32.67, 16.64, 33.92, 9.30, and 28.94 min, respectively. Pinostrobin chalcone, pinocembrin, and tectochrysin are classified in the group of flavonoids. 2,1,3-Benzothiadiazole is a bicyclic aromatic chemical composed of a benzene ring. Phenethyl alcohol is an aromatic alcohol and oleic acid is classified as aliphatic acids [28,29]. Obtained results were similar to the reported findings of other studies, which found aromatic compounds and alcohols in the Iranian different propolis extracts [28–31]. The composition of propolis is drastically affected by its sources, botanical category, geographical regions, and the season of resins collections by the bees [32–34]. In fact, propolis composition, including phenolic compounds, flavonoids, terpenes, and aromatic acids, has main role in its physico-chemical properties and biological activities [30].

### 3.2 Fitting the models

General models using obtained experimental data (Table 1) were generated to determine the correlation between responses and the process parameters. Relatively higher values for the coefficients of models ( $R^2$ ) as shown in

Table 2, which were 91.01, 97.28, 98.31, and 82.66 for particle size, PDI, zeta potential, and antioxidant activity, respectively, indicated the adequacy of the models [24,25,27]. Furthermore, higher lack-of-fit ( $p > 0.05$ ) for all provided models (Table 2) demonstrated desirability of the obtained models. Table 2 also shows the  $p$ -values of all terms for each models, where lower  $p$ -value ( $p < 0.05$ ) indicates high significance of each term. Attained results indicated that processing parameters interaction had significant ( $p < 0.05$ ) effect on particle size, PDI, and antioxidant activity. In addition, results revealed that quadratic term of propolis extract had insignificant effects on the zeta potential and antioxidant activity, while quadratic effect of saponin had insignificant influence on PDI, zeta potential, and antioxidant activity of the prepared propolis nanoemulsions under SW conditions. As attained further achievement, the main term of propolis extract had insignificant influence on particle size and antioxidant activity of the provided nanoemulsions. However, the main term of saponin amount had insignificant effect on particle size of the provided propolis O/W nanoemulsions.

### 3.3 Effect of processing factors on particles size of the made O/W nanoemulsions

Droplet size of the oils has key role on the nanoemulsion characteristics including rheological, optical, biological, and stability. In fact, the colloidal solutions with minimum size of the droplets had higher physical stability [17,35]. Droplet size of the formed propolis O/W nanoemulsions under SW conditions was changed between 145 and 759 nm (Table 1), which was lower than 1,000 nm. This indicated that propolis nanoemulsions could be easily and completely prepared under SW conditions. [25]. Influences of saponin and propolis extract amounts on formed droplet size within nanoemulsions are shown in Figure 2a.

Results illustrated that at constant and low amount of saponin, by rising in propolis extract amount, nanodroplet size decreased, while at high amounts of the used emulsifier, different pattern was observed in the particle size. These opposite manners revealed that interaction term of processing parameters had significant ( $p < 0.05$ ) effect on particle size, as could be reconfirmed by the attained result presented in Table 2. Figure 3a indicates that minimum particles size for the prepared nanoemulsions was achieved at two different areas including propolis extract amounts higher than 0.56 g and amounts of saponin lower than 0.75 g, and two amounts of saponin and propolis extract higher than 1.80 g and lower than 0.16 g, respectively. Attained results revealed that using minimum amounts of saponin, it was possible to surround propolis nanodroplets in the formed nanoemulsion and prevent their movement together. The results were closed into achievement of Moradi and Anarjan [36]. They also found that by decreasing the amounts of emulsifier,  $\alpha$ -tocopherol O/W nanoemulsions with small particle size were prepared [36].

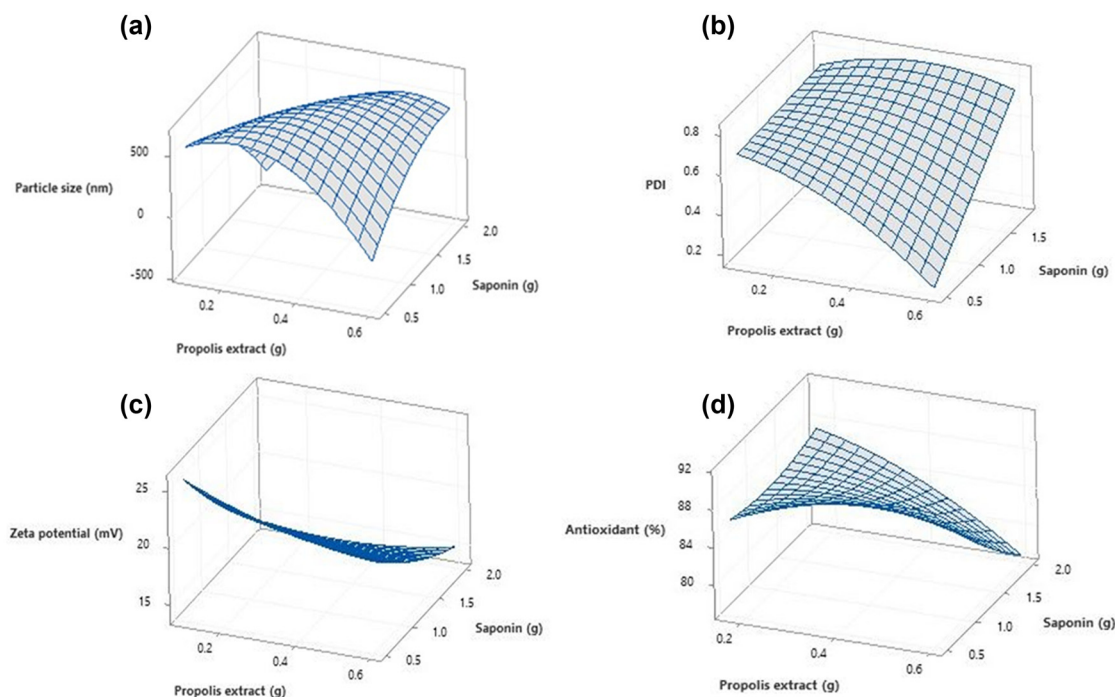
### 3.4 Effect of independent factors on PDI of the resulted O/W nanoemulsions

As shown in Table 1, PDI values of the provided nanoemulsions using SW method were ranged from 0.345 to 0.787. Influences of saponin and propolis extract amounts on the PDI of nanoemulsions are shown in Figure 2b. It is possible to visualize that, at low amounts of emulsifier, by rising the amount of propolis extract, PDI of the nanoemulsions sharply decreased. On the contrary, at higher amounts of saponin, by rising the amount of propolis extract, PDI values did not change significantly. In addition, results illustrated that at higher amounts of propolis extract, by rising the amount of saponin, PDI of the provided nanoemulsions increased. However, at

**Table 2:** Regression coefficients  $R^2$ ,  $R^2$ -adj, and  $p$ -values for the generated models

Regression coefficient	Particle size (nm)	$p$ -value	PDI	$p$ -value	Zeta potential (mV)	$p$ -value	Antioxidant (%)	$p$ -value
$\beta_0$ (constant)	18.40		46.85		77.02		133.90	
$\beta_1$ (main)	0.60	0.569	-6.66	0.001	-3.44	0.018	-1.27	0.250
$\beta_2$ (main)	-2.09	0.081	9.36	0.000	-16.73	0.000	-3.69	0.010
$\beta_{11}$ (quadratic)	-5.75	0.001	-3.40	0.019	1.96	0.107	-1.32	0.235
$\beta_{22}$ (quadratic)	-2.62	0.040	0.05	0.959	1.70	0.150	0.83	0.440
$\beta_{12}$ (interaction)	3.63	0.011	5.40	0.003	2.12	0.087	-2.89	0.028
$R^2$	91.01%		97.28%		98.31%		82.66%	
Lack-of-fit ( $p$ -value)	>0.05		>0.05		>0.05		>0.05	





**Figure 2:** Surface plots for particle size (a), PDI (b), zeta potential (c), and antioxidant activity (d) of the prepared propolis O/W nanoemulsions, as function of amounts of saponin (g) and propolis extract (g).

lower amounts of propolis extract, by rising in amount of saponin, the PDI of the resulted nanoemulsions was constant. These results revealed that the processing factors interaction had significant ( $p < 0.05$ ) effect on PDI of the formed nanoemulsions, as mentioned in Table 2. Figure 3b presents that the small PDI value for the nanoemulsions was obtained at higher amounts of propolis extract than 0.56 g and lower amounts of saponin than 0.60 g. Obtained results were in line with the findings of Moradi and Anarjan that PDI of  $\alpha$ -tocopherol O/W nanoemulsions decreased when the amounts of emulsifier was also decreased [36].

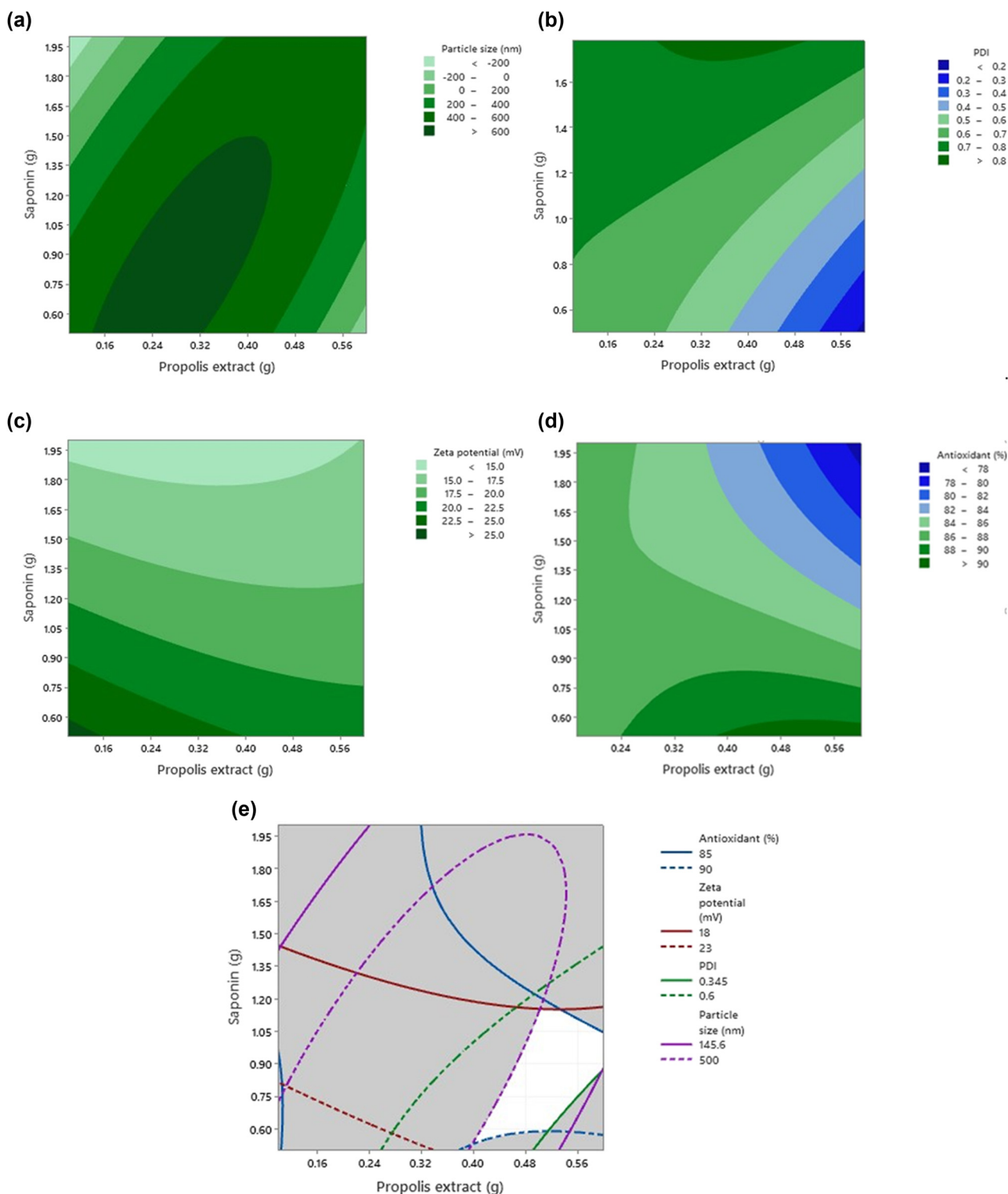
### 3.5 Influences of the processing parameters on zeta potential of the made nanoemulsions

According to Table 1, zeta potential values of the nanoemulsions were ranged from  $-14.0$  to  $-23.0$  mV, which revealed relatively higher stability of the prepared nanoemulsions. Influences of saponin and propolis extract on the zeta potential of the provided nanoemulsions are shown in Figure 2c. Based on the absence of a curvature in this figure, it is possible to understand that independent variables' interactive term had insignificant effect on zeta potential of the provided propolis nanoemulsion.

This was verified high  $p$ -value ( $p > 0.05$ ) of that as mentioned in Table 2. According to Figure 3c, it is possible to recognize that nanoemulsion with highest zeta potential could be provided using low amounts of saponin and propolis extract, under SW conditions. Ahmadi and Jafarizadeh-Malmiri also reported that by decreasing the amounts of emulsifier in production of Thyme O/W nanoemulsions, the zeta potential was increased [10]. It seems that by increasing the amounts of propolis extract, because of agglomeration of the formed propolis nanodroplets in nanoemulsions, their nanodroplet size was increased and their surface was increased, and its charge density was also decreased (Figure 3c).

### 3.6 Influence of processing parameters on antioxidant activity of the made nanoemulsions

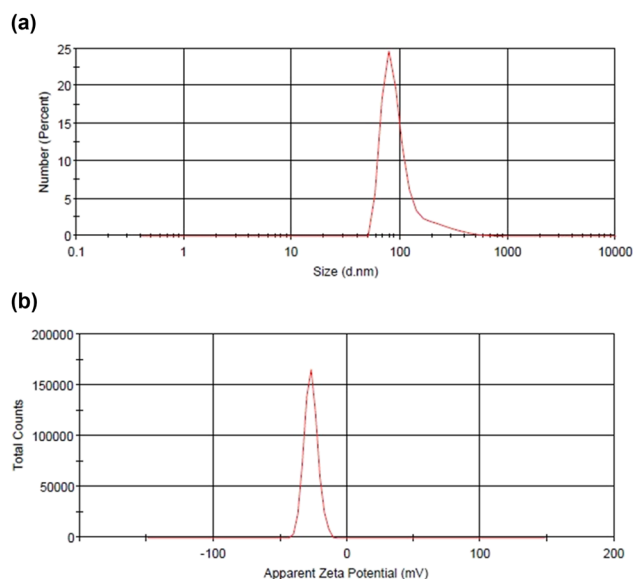
Propolis is a lipophilic substance and because of its antioxidant activity has been widely used in food, healing, and numerous medicinal areas [37]. Experimental values of the antioxidant activity for the made propolis nanoemulsions varied from 79.8% to 89.1% (Table 1). Influences of the processing factors on antioxidant activity of the produced propolis nanoemulsion are shown in Figures 2 and 3d. Based on Figure 2d, at fixed and low mounts of the



**Figure 3:** Contour plots for particle size (a), PDI (b), zeta potential (c), antioxidant activity (d), and overlaid contour plot (e) of the prepared propolis O/W nanoemulsions, as function of amounts of saponin (g) and propolis extract (g).

emulsifier, by rising the amount of propolis extract, antioxidant activity of the formed nanoemulsions was constant. However, at fixed and high amounts of the emulsifier, by

rising the amount of propolis extract, the antioxidant activity of the resulted propolis nanoemulsion decreased. As shown in Figure 2d, presence of curvature revealed that interaction

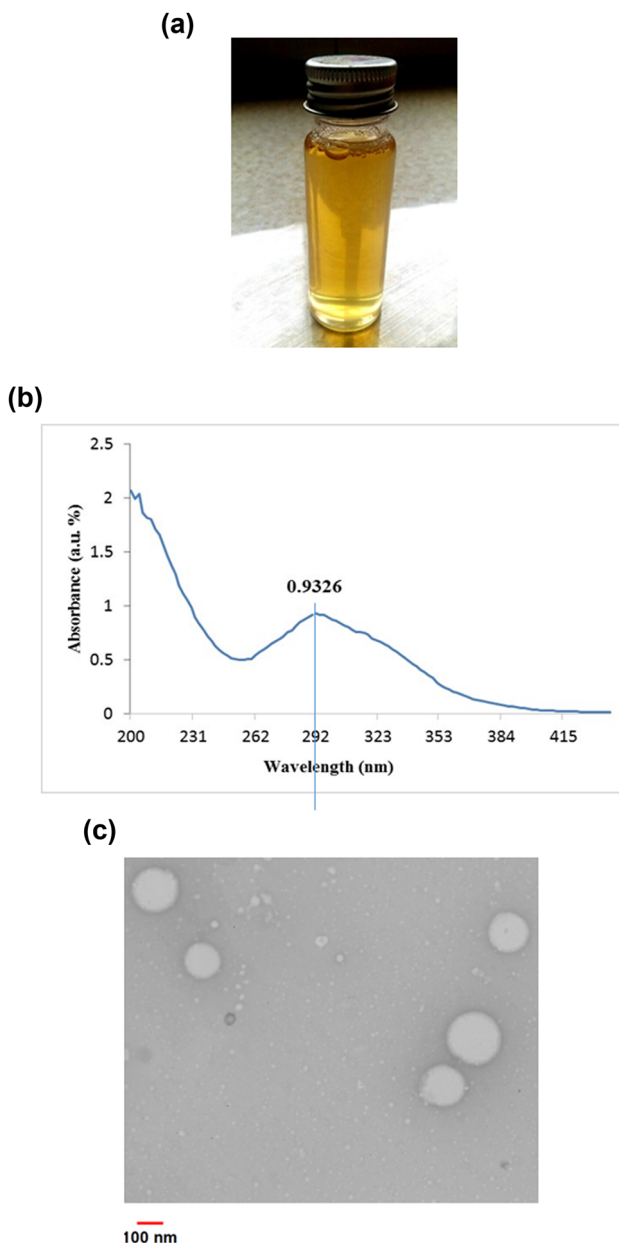


**Figure 4:** Particle size distribution (a) and zeta potential distribution (b) of the prepared propolis O/W nanoemulsion using obtained optimum production conditions.

term of the processing parameters had significant ( $p < 0.05$ ) effect on antioxidant activity of the prepared O/W nanoemulsions. As shown in Figure 3d, the highest antioxidant activity was attained in the resulted propolis nanoemulsion that were made using lower and higher amounts of saponin and propolis extract, respectively. Achieved results were consistent with the results of Mehmood et al. [38], who reported that in prepared olive oil-based O/W nanoemulsions by increasing the amounts of emulsifier in the nanoemulsions, the antioxidant activity of the formed colloidal solutions decreased. Antioxidant activity decreased in larger surface area possibly because of more contact between nanoparticles and aqueous phase. Propolis is rich in phenolic compounds, which may act as natural antioxidants [39]. In fact, because of the presence of different groups of phenolic acids and flavonoids in propolis extract, which are its main components with antioxidant activity, its antioxidant activity is directly related to their concentration [40,41].

### 3.7 Optimization of independent variables

Overlaid contour plot based on the generated models show graphical optimization (Figure 3e). White colored area in Figure 3e, was related to the optimum amounts of two selected variables, for preparation of nanoemulsions with desirable characteristics. Attained results based on numerical optimization also revealed that provided propolis O/W nanoemulsions using SW, 0.53 g propolis extract, and 0.50 g saponin had small particle size and PDI, high



**Figure 5:** (a) Appearance, (b) UV-Vis spectrum, and (c) TEM image of the prepared propolis O/W nanoemulsion using obtained optimum production conditions.

zeta potential, and antioxidant activity of 144.06 nm, 0.286, -21.71 mV, and 90.86%, respectively.

### 3.8 Characteristics of the formed propolis nanoemulsion using optimal processing conditions

Based on the obtained optimum conditions for the preparation of propolis O/W nanoemulsion, nanoemulsions



were experimentally prepared and their physico-chemical attributes were assessed. DLS analysis indicated that particle size, PDI, and zeta potential values of the resulted nanoemulsion were 134.6 nm, 0.301, and  $-22.8$  mV, respectively. Furthermore, there were insignificant differences between the values of the experimental and predicted responses. Particle size and zeta potential distributions for the produced nanoemulsion are shown in Figure 4a and b. Based on Figure 4a, the narrow and sharp highlighted peak revealed that monodispersed propolis nanodroplets were made in the colloidal solution with small PDI value [22,25]. Furthermore, according to Figure 4b, high zeta potential peak indicated the high stability of the nanoemulsion, using attained optimal processing conditions [22].

Turbidity and appearance of the provided propolis nanoemulsion were shown in Figure 5a. Turbidity has been known as a main index related to the nanoemulsions quality. Achieved results indicated that the made propolis nanoemulsion with SW and using optimum operation conditions was transparent and had turbidity value of 0.08 a.u. Differences in droplet size have a great effect on the stability of emulsion systems, so that the size of fine droplets causes greater transparency and stability of the produced nanoemulsions [42].

Formation of propolis O/W nanoemulsion using propolis extract and saponin emulsifier at obtained optimum operation conditions was reconfirmed based on the SPR characteristic of the made nanodroplets. According to Figure 5b, the maximum absorbance peak ( $\lambda_{\max}$ ) of the prepared nanoemulsion was observed at wavelength of 292 nm, which was centered in the range of 290–300 nm.

Morphological attributes of the provided propolis O/W nanoemulsion using SW are shown in Figure 5c. As visually could be seen in this figure, the prepared nanoemulsions contained spherical propolis nanodroplets, which revealed low surface energy amount of droplets and their highest stability. Furthermore, TEM analysis shows that the mean droplet size of the propolis in the nanoemulsion was 80 nm.

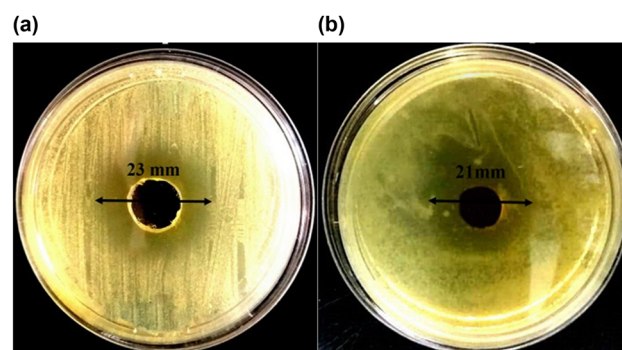
In addition, the results illustrated that the provided propolis nanoemulsion used saponin and the prepared propolis extract had antioxidant activity of 91.1%, 10.9%, and 94.5%, respectively. Small value for antioxidant activity of the provided propolis nanoemulsion might be related to low concentration of propolis extract (0.53 g), which was presented in the 45 mL of produced nanoemulsion. Obtained results were in line with finding of Ahmadi and Jafarizadeh-Malmiri [10]. They also reported that the antioxidant activity of the made thyme O/W nanoemulsion using saponin and SW was lower than that of the pure thyme oil.

### 3.9 Antibacterial activity of the made propolis nanoemulsion

Bactericidal activities of the provided propolis O/W nanoemulsion at obtained optimum operation conditions, propolis extract, and saponin toward *S. aureus* and *E. coli* are shown in Figure 6. Attained results demonstrated that the produced nanoemulsion had high bactericidal effect toward both *S. aureus* (23 mm) and *E. coli* (21 mm), and this effect on *S. aureus* was higher than that of on *E. coli*. Obtained results can be correlated into the differences between cell wall structure of gram-negative and gram-positive bacteria strains. In fact, on the surface of peptidoglycan layer in gram-negative bacteria, there are lipoproteins, purins, and proteins which can strongly protect the peptidoglycan layer against antibiotics [43–45].

## 4 Conclusion

The results of the present study revealed that application of SW in nanoemulsion preparation could be effectively used to intensify the process, where in this process there is no need to use chemical solvent as an inorganic phase, and further process to remove residual solvent from the provided nanoparticles. Furthermore, using SW can effectively reduce using emulsifier in the nanoemulsion preparation procedure, which makes the process cost effective. Saponin as natural emulsifier could be easily used in the preparation of propolis O/W nanoemulsion with desirable physico-chemical and biological attributes. Finally, RSM could efficiently model the nanoemulsion preparation procedure, correlated the responses with the independent variables, and optimized the processing conditions.



**Figure 6:** Created zones of inhibition of the prepared propolis O/W nanoemulsion using obtained optimum operation conditions *S. aureus* (a) and *E. coli* (b) incubated at 37°C for 24 h.

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