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Clove oil nanoemulsion as an effective antibacterial agent: Taguchi optimization method

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ABSTRACT

Clove oil is an essential oil used as a biodegradable antibacterial agent in food and pharmaceutical products. Emulsions are used to stabilize and increase the antimicrobial efficacy of oils in aqueous solutions. Smaller droplets provide better distribution of the oil. In this study, we formulate clove oil in aqueous solution by nanoemulsion. The aim of this study was to optimize key parameters in the preparation of clove oil nanoemulsion to obtain stable emulsions with less than 50 nm droplet size. The evaluated parameters include the concentration of clove oil, concentration of emulsifier, hydrophilic–lipophilic balance (HLB) number, and ultrasonication time. We determined the following response parameters: droplet size, polydispersity index, and zeta potential, and we aimed to achieve <50 nm droplet size with low polydispersity index and a highly negative zeta potential. The optimization and evaluation was obtained via Taguchi method. Taguchi method identified the concentration of clove oil and the HLB as the most affecting factors in the preparation of nanoemulsions. The optimum conditions for the preparation of nanoemuslion such as mean droplet size, polydispersity index, and zeta potential were 50 nm, 0.49 and −40.7 mV, respectively. This formulation of clove oil resulted in minimum inhibitory concentration against *Escherichia coli* and *Bacillus cereus* of 16 and 32 μg/ml, respectively, and minimal bactericidal concentrations of 16 and 64 μg/ml, respectively.

Keywords: Antibacterial; Clove oil; Droplet size; Nanoemulsion; Taguchi optimization

1. Introduction

Nowadays, environmental pollutions caused by excessive use of pesticides have brought scientists and public special attention. Applications of effective green pesticides in agricultural production fields are highly recommended to prevent crop yield losses and obtain high production yield. Synthetic chemicals used as pesticides may cause serious side effect, such as penetration of hazardous chemical into surface water bodies and create environmental complication due to toxicity of chemicals used [1,2].

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Nanoemulsions are one of the emulsion types that can be prepared from oil, water, and emulsifier with particle sizes ranged in 10–100 nm [3]. The biggest difference between nano and conventional emulsions is the size of droplets. Nanoemulsion possesses several advantages, such as high physical stability (stability to gravitational forces, flocculation and coalescence), high bioavailability, and low turbidity, which make them more attractive systems for food applications [4–6], cosmetics [7], and pharmaceuticals [8]. Apart from droplet size, coalescence also depends on other environmental factors, such as the type of emulsifiers used (ionic/nonionic), pH, and storage temperature. Generally, oil in water nanoemulsions acts as a carrier of bioactive compounds for instance essential oil (dispersed phase) in water (continuous phase). Essential oils are potential sources of novel antimicrobial compounds; especially used against pathogenic bacteria [9]. Generally, nanoemulsion of natural oil in water cannot be easily separated from aqueous phase [10]. Therefore, they can be formulated with different bioactive agents, such as drugs in pharmaceutical industry [5], as a flavor [11], solubilizing agent, water-insoluble compounds in agrochemical industry [5,12], lotion for skincare in cosmetics [7] and also used as antimicrobial agents in food industry [13].

Clove oil has biological activities, such as antibacterial, antifungal, insecticidal, and antioxidant properties. It is traditionally used as a flavor and antimicrobial agent in food [13,14]. Eugenol is the major constituent of clove essential oil; it is a phenolic compound with strong biological and antimicrobial activities. It can denature protein and react with phospholipids in cell membrane. Also, it can change cell membrane permeability and inhibit a great number of Gram-negative and Gram-positive bacteria as well as various types of yeast [15,16].

Ultrasonic emulsification is an energy-efficient method to develop nanoemulsions. This method is documented as a fast and efficient technique for formulating stable nanoemulsions with very small droplet size and low polydispersity [8,17]. It takes advantage of sound waves to generate powerful shock waves and create acoustic cavitation to disrupt the coarse droplets with the aid of an ultrasonic probe homogenizer [4,8,18]. This method is preferable over mechanical methods due to its lower energy consumption (through heat loss) and use of small amounts of emulsifiers. Ultrasonic emulsions have more uniform droplet size and are more stable compared to emulsions achieved by mechanical agitation [18–20].

Taguchi design method is a fractional factorial design which uses an orthogonal array (OA) that can greatly reduce the number of experiments [21]. In addition, analysis of variance (ANOVA) can estimate the effect of a factor on the characteristic properties. The variability is decisive for choosing the optimal condition. Our aim is to determine the smallest droplet size with high stability. The variability is expressed by signal-to-noise (S/N) ratio. This is an important factor to simulate and analyze the influence of multiple control factors on performance as well as to investigate the influence of individual factors to determine the most effective parameters [22].

The nanoemulsion clove oil droplet size of less than 50 nm was not reported [23,24]. The objective of present work was to optimize process parameters in the preparation of clove oil nanoemulsion by means of oil and emulsifier concentrations, sonication time, and hydrophilic–lipophilic balance (HLB) number. The clove oil nanoemulsion was tested as antibacterial agent against *Escherichia coli* and *Bacillus cereus.*

2. Materials and methods

2.1. Materials

Clove oil (C8392) was purchased from Sigma–Aldrich® (St. Louis, MO, USA); the nonionic surfactants of polyethylene sorbitan monooleate (822187, Tween® 80) synthetic grade and sorbitan monooleate (840123, Span® 80) synthetic grade were purchased from Merck-Millipore® (Darmstadt, Germany); water used in all the experiments was purified with a Milli-Q system consisting of filtration through 0.2 μm filters (Milipore Co., Bedford, MA, USA). For antibacterial testing; microbiological grade of peptone from casein (70171), peptone from soybean (70178), and agar powder (05040) were purchased from Fluka® Analytical (Dresden, Germany). In addition, dipotassium hydrogen phosphate (60353) and sodium chloride (S9888) were supplied from Sigma–Aldrich® (St. Louis, MO, USA). D (+)-glucose (108337) was obtained from Merck-Millipore® (Darmstadt, Germany).

2.2. Antimicrobial activity assay

Gram-negative bacteria, *E. coli* K12 (LZB035; Kent, UK) and Gram-positive bacteria, *B. cereus* (ATCC 10987) were maintained on tryptic soy agar (TSA) plates and single colonies were inoculated and cultured overnight in tryptic soy broth (TSB) (17 g/l peptone–casein, 3 g/l peptone–soybean, 2.5 g/l dipotassium hydrogen phosphate, 2.5 g/l glucose, and 5 g/l sodium chloride) at 37˚C and agitated at 120 rpm. Trypticase soy agar (TSA plate) was used as solid culture with 1.5% agar added to the TSB media and then plates were stored at 4˚C.

The estimation of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of optimum formula of clove oil nanoemulsion were measured by the broth microdilution method. Briefly, overnight cultures were diluted in fresh TSB to 10^7 CFU/ml, and diluted 10-fold into 200 μl TSB in sterile 96-well microtiter plates (NUN-161093, Nunclon™) prepared with a concentration of the clove oil nanoemulsion ranging from 1 to 1,024 μg/ml after addition of the inoculum to the wells. Nanoemulsion-free wells served as positive controls (growth control) and bacteria-free well served as blanks. Microtiter plates were then incubated statically at 37˚C for 24 h, and then optical density at 600 nm (OD_{600}) was determined using a microtiter plate reader (Power Wave™ XS2; BioTek®, Vermont, USA). The MIC was defined as the lowest concentration of the clove oil nanoemulsion at which an increase in OD_{600} compared to the blanks could not be detected. The MBC was determined by transferring 10 μl of each suspension from wells with no apparent growth to TSA and examining growth after incubation at 37˚C for 24 h. The MBC was defined as the lowest concentration of the clove oil nanoemulsion resulting in no detection of viable cells, corresponding to kill >99.99% of the bacteria. Each experiment was replicated for four times.

2.3. HLB number

Nonionic surfactants consist of molecules that combine both hydrophilic and lipophilic groups. They are classified according to the balance of the size and strength of these two opposing groups which is called the HLB, and assigned a HLB number to each of these molecules. In general, the HLB number is in a wide range from 0 (hydrophobic end) to 20 (hydrophilic end) such that high HLB surfactants are used for making oil in water emulsions.

Tween® 80 (HLB value of 15.0) and Span® 80 (HLB value of 4.3) as mixtures were selected for the preparation of emulsions; because of their high degree of compatibility with other ingredients, chemically stable, and having low toxicity. Fig. 1 demonstrates the hydrophilic and hydrophobic tails of Tween® 80 and Span® 80, respectively. Span® 80 as a viscous, lipophilic, emulsifying liquid agent is often used to form water in oil emulsions. Tween® 80, hydrophilic in nature, is a derivative of Span® 80 due to hydroxyl functional groups on sorbitan ring are replaced by the bulky polyoxyethylene groups. Such substitutions make Tween® 80 more soluble in water, which then easily formed oil in water emulsions. Furthermore, Tween® 80 and Span® 80 are generally considered as safe additives in pharmaceutical, cosmetic, and food products. Nonionic surfactants as uncharged molecules are also known as safe and biocompatible products; they are not affected by pH changes in media [25–27].

Use of single surfactant was tested, due to low or high HLB values, and also based on zeta potential data the droplets size was not quite stable. A blend of hydrophilic and lipophilic surfactants is required to obtain longer stability of the dispersion phase at the lowest concentration levels. Also, a blend of surfactants with an HLB that matches with the oil phase has to provide high solubilization and stability of the dispersion system. Therefore, the selection of surfactant blends at low and high HLB matches the HLB of oil which is important in the formulation of a colloidal system. In this research, the HLB number system was used to optimize a compatible nonionic mixture of surfactants and calculate the HLB number of mixed surfactants system by the following expression:

$$HLB = \frac{m_A \times HLB_A + m_B \times HLB_B}{m_A + m_B} \tag{1}$$

where m_A (g) and m_B (g) are the mass of the surfactants A and B, respectively. HLB_A and HLB_B are the HLB number of the surfactants A and B, respectively [28].

2.4. Taguchi design of the experiment

The Taguchi design method applies fractional factorial test designs called OAs that serve to reduce the number of experiments. The selection of a suitable OA depends on the number of control factors and their levels. Using OA design can estimate multiple process variables which is simultaneously affecting on the performance characteristic, while minimizing the number of test runs. For example, for four parameters at three levels, the traditional full factorial design would require 3^4 or 81 experiments. In the Taguchi L9 OA, however, the required experiments are only 9. To accomplish such goal, Taguchi L9 OA design (four factors with 3-level design) was used to determine and optimize the instrumental parameters to consider very small droplet size diameter and long storage stability and small polydispersity. The design of experiment (DOE) using the Taguchi method provides a simple, efficient, and systematic approach to determine the optimum conditions [29–31].

2.4.1. Effective factors and levels

There are four key factors in the preparation of clove oil nanoemulsion procedure which include the

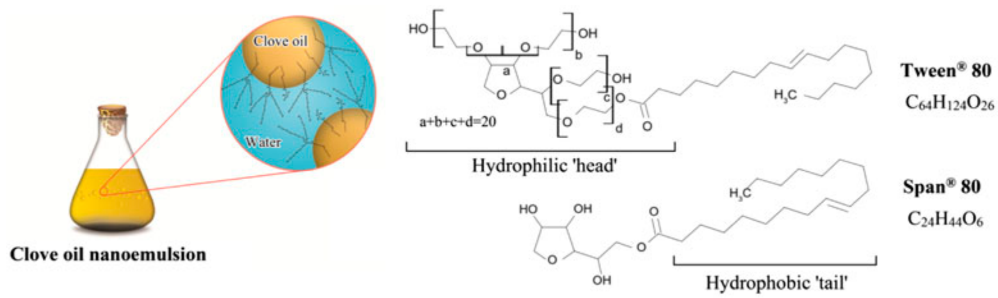

Fig. 1. Specification of surfactants.

oil concentration (*A*), emulsifier concentration (*B*), HLB number (*C*), and ultrasonic emulsification time (*D*). These four factors, each at three levels, are summarized in Table 1.

2.4.2. Selection of OA and factor assignments

In this research, L9 OA (four parameters, in three levels) was used. To observe the data reliably on experiment, droplet size (nm), polydispersity index (PDI), and zeta potential (mV) data were repeated three times with the same conditions. L9 OA was used to determine the instrumental parameters shown in Table 2. Each row in this table represents a trial condition with the level of factors.

For analysis of the results and optimized conditions for setting the control factors, QUALITEK-4 (QT4) software was used. QT4 Version 4.75 is the MS-Windows 7 version software for automatic design and analysis of Taguchi experiments.

2.4.3. Signal-to-noise ratio

In the Taguchi method "signal" and "noise" for output characteristics and the signal/noise ratio (S/N) represent the desirable signal value and the undesirable noise value, respectively. The ratio is used to transform the quality characteristics [32]. The equation of S/N ratio depends on the criteria used for optimization of the quality characteristics. In this research, the results of nanoemulsion of mean droplets size, PDI, and zeta potential values were used to calculate the corresponding S/N ratio, and the equation is stated as follows:

$$S/N = -10 \log\left(\frac{1}{n}\sum Y^2\right) \tag{2}$$

where n is the number of tests and Y is the response factor.

The S/N ratio mean values for samples at each set of experimental conditions for each process parameter variables are obtained. Based on the orthogonal experimental design, the effect of each process parameter on S/N ratio at different levels for each sample is properly reported.

2.4.4. Statistical analysis

ANOVA was performed in order to determine significant differences among the factors. It evaluates significance of the controlling factors by calculating the *F*-ratio (variance ratio) and the percentage contribution by each of the design parameters and error. Sum of squares (*SS*), degrees of freedom (*DOF*), mean of square (MS), and associated *F*-test of significance (5%) (*F*) can be calculated as follows:

$$SS_A = \left(\sum_{i=1}^{k_A} \frac{A_i^2}{n_{A_i}}\right) - \frac{T^2}{N} \tag{3}$$

Table 1
Selected process parameters and respective levels in the experimental design

Process parameters	Level 1	Level 2	Level 3
(A) Clove oil concentration (wt%)	2.5	7.5	15.0
(B) Emulsifier concentration (wt%)	2.5	5.0	7.5
(C) Mixed HLB number	9.0	12.0	15.0
(D) Ultrasonication time (s)	150	300	450

Table 2
Experimental layout by Taguchi L9 OA design

Experimental run number	Parameters			
	Clove oil concentration (wt%)	Emulsifier concentration (wt%)	Mixed HLB number	Ultrasonication time (s)
1	2.5	2.5	9.0	150
2	2.5	5.0	12.0	300
3	2.5	7.5	15.0	450
4	7.5	2.5	12.0	450
5	7.5	5.0	15.0	150
6	7.5	7.5	9.0	300
7	15.0	2.5	15.0	300
8	15.0	5.0	9.0	450
9	15.0	7.5	12.0	150

where k_A is the number of the levels of factor A, n_{A_i} is the number of all observations at level i of factor A, A_i is the sum of all observations of level i of factor A, and T is the sum of all observations. SS of error is computed by the following equation:

$$SS_e = SS_T - (SS_A + SS_B + \cdots) \tag{4}$$

where SS_T is the total SS:

$$SS_T = \sum_{i=1}^{N} y_i^2 - \frac{T^2}{N} \tag{5}$$

where y_i is the observation of i. MS is calculated by dividing the SS by the DOF. DOF_A is estimated by $DOF_A = k_A - 1$. F-value is calculated as follows:

$$F_A = \frac{MS_A}{MS_e} \tag{6}$$

MS_e is the variance of error.

The percentage contribution for each factor is defined as the portion of a total observed variance in the experiment for each significant factor. The greater the value the more it contributes to the final results. Quantitative evaluation can be achieved using percentage contribution (P (%)). It is calculated by dividing the source's net variation by SS_T, which is given as follows:

$$P\ (\%) = \frac{SS_A - (DOF_A \times MS_e)}{SS_T} \times 100 \tag{7}$$

2.5. Clove oil nanoemulsion

In order to obtain clove oil emulsion in nano droplets size, the number of blends of different weight percentages of Span® 80 (HLB = 4.3) and Tween® 80 (HLB = 15) for various HLB number on experimental design were prepared. These variables were determined from Eq. (1) as indicated in Table 3. For example, surfactant mixing ratio to obtain the HLB no. 9, 56 wt% of Span® 80 with 44 wt% of Tween® 80 were mixed.

Based on Taguchi design, sample preparation with different concentration of clove oil and surfactants blended with Span® 80 and Tween® 80 (based on different blends summarized in Table 3) to Milli-Q water as the aqueous phase in a test tube. Then, all samples were premixed by vortex mixer IKA®-MS2 Minishaker (IKA Labortechnik, Staufen, Germany) for 1 min; further emulsification by sonication was performed for 3 times using an ultrasonic homogenizer with a power of 70 W at 20 kHz using Sonopuls HD 2070 (Bandelin Electronic, Berlin, Germany) equipped with a 3-mm diameter Sonotrode probe made of titanium. In order to keep the temperature below 30°C, the emulsions were maintained in an iced water bath. Then the mean droplet diameter, PDI, and zeta potential of all the samples were determined. Fig. 2 presents the schematic diagram of the experimental set up for preparation of nanoemulsions.

Table 3
Ratio of mixed surfactants in preparation of mixed HLB number based on Eq. (1)

		Mixed HLB number		
		9	12	15
$HLB_{A(\text{Span® 80})} = 4.3$	$m_A =$	56	28	0
$HLB_{B(\text{Tween® 80})} = 15$	$m_B =$	44	72	100

2.6. Droplet size and PDI analysis

One of the most important physical characteristics of a nanoemulsion is the droplets size distribution. Dynamic light scattering (DLS) with a Malvern Zetasizer® Nano ZS (model ZEN 3600, Malvern Instruments, Worcestershire, UK) operating at a fixed scattered angle of 173˚ was used for all the samples of oil in water emulsions. The mean droplet diameter (Z-average) of the samples and polydispersity indices were calculated from the droplet size distribution. Z-average size (also known as the "cumulants mean") is the size to use if a number is required for quality control purposes. In DLS, this is the most important and stable number produced by Z-average technique. The software used to collect and analyze the data was the Zetasizer® Software (version 7.03). These parameters were obtained from the autocorrelation function using the "general purpose mode" analysis model. Also, measurements were controlled at 25℃; each measurement was repeated for three times and the average value was reported.

Refractive index (RI) value of 1.59 was used for emulsion solution with several concentration of clove oil. For the sample RI, it is only required to display or print the result as a volume distribution. It is not required for calculation of the Z-average size.

2.7. Zeta potential measurements

Zeta potentials of nanoemulsion samples were measured in a disposable folded capillary cell model DTS1070 (Malvern Instruments, Worcestershire, UK) at 25℃ using the Zetasizer® Nano ZS (model ZEN 3600, Malvern Instruments, Worcestershire, UK) were controlled through the Zetasizer® Software (version 7.03). Before any measurements, cells were allowed to equilibrate at 25℃ for 120 s. All experiments were performed in triplicates. The average and standard deviation of the zeta potential were calculated from three separately independent prepared samples measurements.

3. Results and discussion

There is no scientific literature to demonstrate preparation and optimization of clove oil nanoemulsion by ultrasonic emulsification using Taguchi method. Also, the nanoemulsion droplet size of less than 50 nm has not been reported in the literature. In this work, the responses of 3 variables known as least average of droplet size (nm) (Y_1); PDI (Y_2) and zeta potential (mV) (Y_3) for the desired experimental outcome were 28.8 nm, 0.38, and −40.1 mV, respectively (Table 4).

3.1. Signal-to-noise ratio

The mean value of the S/N ratio in decibel (dB) at different levels of the process parameter for mean droplet size (nm), PDI, and zeta potential (mV) are illustrated S/N graphs in Fig. 3(a)–(c), respectively. Basically, it is defined that smaller S/N ratio is the better quality characteristic for the nanoemulsion.

3.2. ANOVA

The purpose of ANOVA is to investigate which process parameter significantly affected the properties of clove oil nanoemulsion such as, nanoemulsion mean diameter under 50 nm with zeta potential below −30 mV (high stability), and lowest PDI. Table 5 contains the ANOVA for (a) mean droplet size (nm), (b) PDI and (c) zeta potential (mV). In this table, the rows marked as "Error" that refer to errors caused by uncontrollable factors (noise) that are not included in the experiment and the experimental error. In general,

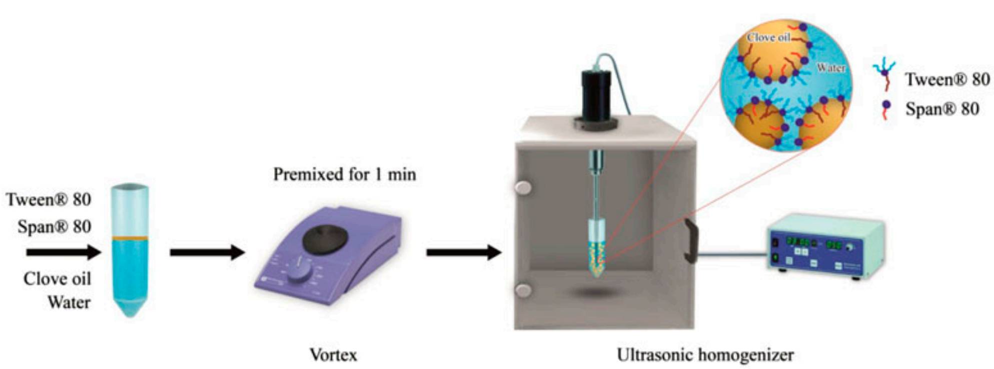

Fig. 2. Schematic diagram of the experimental set up for preparation of clove oil nanoemulsion.

Table 4
Responses of various nanoemulsions of clove oil (mean ± SD, replicated 3 times)

	Responses		
Experimental run number	Y_1	Y_2	Y_3
1	127.8 ± 2.3	0.46 ± 0.03	−30.9 ± 0.60
2	134.6 ± 2.6	0.38 ± 0.06	−13.0 ± 0.44
3	310.3 ± 6.2	0.56 ± 0.02	−10.4 ± 0.21
4	60.5 ± 1.7	0.52 ± 0.02	−29.5 ± 0.67
5	57.7 ± 1.5	0.55 ± 0.02	−16.3 ± 0.57
6	49.1 ± 2.3	0.50 ± 0.01	−29.5 ± 0.95
7	47.0 ± 1.3	0.49 ± 0.01	−32.4 ± 0.15
8	34.0 ± 1.2	0.49 ± 0.05	−40.1 ± 1.04
9	28.8 ± 1.6	0.48 ± 0.03	−17.3 ± 0.35

Notes: Y_1 (mean droplet size (nm)); Y_2 (PDI); Y_3 (zeta potential (mV)).

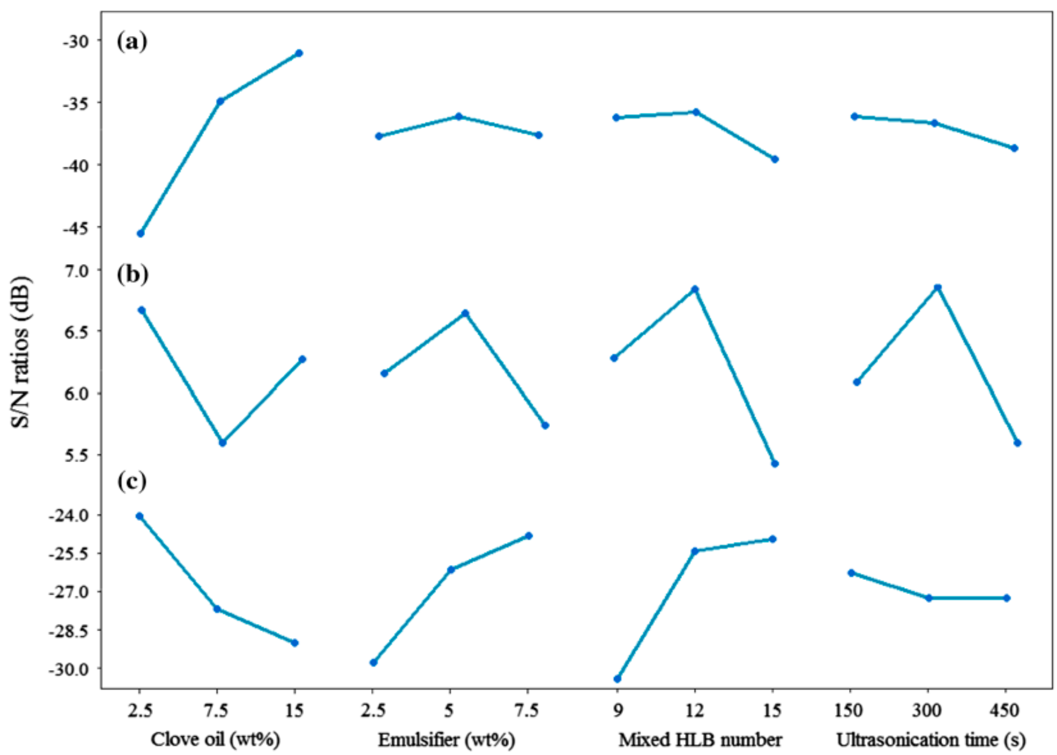

Fig. 3. S/N ratio graphs for (a) mean droplet size (nm), (b) PDI, and (c) zeta potential (mV).

the value should be less than 50%, otherwise, results would not be reliable. Here, the calculated error was about 0.1% for mean droplet size experiments, 16.7% for PDI, and 0.5% for zeta potential experiments. It can be seen that these are significantly under the limit. It means that nearly all important and effective factors have been considered and that errors in the experiments are not significant.

Fig. 4 shows the contribution of clove oil concentration which has highly significant effect on nanoemulsions' performance indicators; especially in the case of mean droplet size of nanoemulsions that had superior effects. On the contrary, the effect of emulsifier concentration was the lowest among the other experimental factors. In addition, the effect of mixed HLB number was considered on zeta potential

Table 5
ANOVA for (a) mean droplet size (nm), (b) PDI, and (c) zeta potential (mV)

Factors	*DOF* (f)	Sum of sqrs. (*SS*)	Variance (*V*)	*F*-ratio (*F*)	Percent *P* (%)
(a) Mean droplet size (nm)					
Clove oil concentration (wt%)	2	127,329.1	63,670.4	10,914.7	66.2
Emulsifier concentration (wt%)	2	16,547.5	8,279.6	1,419.3	8.6
Mixed HLB number	2	26,093.6	13,052.6	2,237.5	13.5
Ultrasonication time (s)	2	22,261.8	11,136.8	1,909.1	11.6
Error	18	105.0	0.0		0.1
Total	26	192,383.7			100.0%
(b) PDI					
Clove oil concentration (wt%)	2	0.016	0.008	16.224	19.612
Emulsifier concentration (wt%)	2	0.008	0.004	8.297	9.400
Mixed HLB number	2	0.024	0.012	24.896	30.784
Ultrasonication time (s)	2	0.019	0.009	19.207	23.455
Error	18	0.008	0.000		16.749
Total	26	0.077			100.00%
(c) Zeta potential (mV)					
Clove oil concentration (wt%)	2	633.6	316.8	720.5	24.8
Emulsifier concentration (wt%)	2	654.4	327.2	744.3	25.7
Mixed HLB number	2	1,128.2	564.1	1,283.1	44.2
Ultrasonication time (s)	2	123.9	61.9	140.9	4.8
Error	18	7.9	0.4		0.5
Total	26	2,548.0			100.0%

and PDI. The obtained data showed that the ultrasonication time was another effective parameter on PDI and particle size of nanoemulsion.

ANOVA may be used to estimate the process performance at optimum conditions. In Taguchi method, optimum conditions are those that may result in the smallest S/N ratio. Levels with a low S/N ratio are those that represent the optimum conditions for a considered factor. Based on ANOVA results, the process parameter for the optimal clove oil nanoemulsion properties are not the same. The results of optimum conditions of mean droplet size, PDI, and zeta potential are listed in Table 6. The S/N ratio at optimum condition and the expected condition has resulted in minimum droplet size. The processing conditions for attaining optimal concentration of clove oil (A) at level 3, concentration of emulsifier amount (B) at level 2;

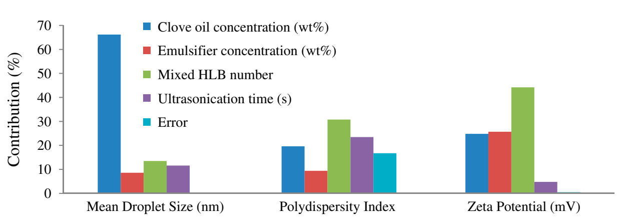

Fig. 4. Results of ANOVA for test series, contribution of each factor on the performance indicators.

HLB number (C), and ultrasonication time (D) at level 1, are found to be optimum. At the optimum conditions predicted by Taguchi method, the S/N ratio improved to about −29.5 dB. Thus, the expected value of the droplet size at optimum conditions was 30 nm.

Table 6 part "b" shows that the predicted conditions to give minimum PDI are similar to the experimental conditions of run 2 (Table 2). At the optimum conditions expected by Taguchi method, the S/N ratio was defined as 0.374 dB. Thus, the obtained PDI based on the S/N 0.374 is approximately 0.38. The reason for this improvement is that the levels (experimental conditions) in optimum conditions are those with the smallest S/N ratio.

The expected S/N ratios for zeta potential at optimum condition, such as concentration of clove oil amount (A) and ultrasonication time (D) at level 3, and concentration of emulsifier amount (B), and HLB number (C) at level 1was about −47.9 mV (Table 6 part c).

Table 7 shows the specific optimum conditions for clove oil nanoemulsion. In fact, our goal is at optimal conditions to obtain droplet size of 50 nm with minimum PDI and zeta potential (mV).

In comparison of actual experimental data with predicted values which were projected by Taguchi method using the information defined in final step of optimization, the experimental values were quite close

Table 6
Optimum conditions for mean droplet size, PDI and zeta potential

	(a) Mean droplet size (nm)		(b) PDI		(c) Zeta potential (mV)	
Factors	Level	Description	Level	Description	Level	Description
Clove oil concentration (wt%)	3	15	1	2.5	3	15
Emulsifier concentration (wt%)	2	5	2	5.0	1	2.5
Mixed HLB number	1	9	2	12.0	1	9
Ultrasonication time (s)	1	150	2	300	3	150
Expected result at optimum condition	30		0.374		−47.88	

and comparable to the predicted values (Table 8). The predicted values for zeta potential, mean droplet size, and PDI were −47.9 mV, 47.75 nm, and 0.509, respectively. In addition, the experimental values for zeta potential, droplet size, and PDI were −40.7 mV, 50 nm and 0.496, respectively. The finalized formulations at optimal conditions were utilized for MIC and MBC tests.

3.3. Validation experiment for Taguchi optimal prediction compare to experimental data

Validation experiments are required, in order to verify the expected results of clove oil nanoemulsion obtained at the optimal level for processing factors. If the observed results in the confirmation experiments are within the confidence limit (5%), then the projected results, according to Taguchi method outputs are acceptable. Validation experiments were performed for optimum levels. The observed values of mean droplet size (level: *A*3, *B*2, *C*1, and *D*1), PDI (level: *A*1, *B*2, *C*2, and *D*2) and zeta potential (level: *A*3, *B*1, *C*1, and *D*3) were 32.2 ± 2.3 nm, 0.38 ± 0.06 and −40.7 ± 2.04 mV, respectively.

The mean values of droplet size, PDI, and zeta potential at optimum condition were projected by Taguchi L9 OA design, as 30 nm, 0.374 and −47.9 mV, respectively.

The data shows the determined range of desirability of data which is about 85% confidence interval. It can be seen that the clove oil nanoemulsion is greatly improved at the defined levels. The zeta potential of optimum clove nanoemulsion has suitable physical stability of formulation.

As particles with potential higher than +30 mV and lower than −30 mV are considered stable, the high stability of nanoemulsions at high zeta potential value is attributed to presence of high surface charge. It reduces the risk of coagulation due to electrostatic repulsion between particles bearing same electric charges, and makes the system stable for longer time and allows easy re-dispersion.

3.4. Bacteriostatic and bactericidal activity of optimum formula of clove oil nanoemulsion

Clove oil nanoemulsions were slightly more potent against *E. coli* compared to *B. cereus*. The MIC and MBC concentrations on *E. coli* and *B. cereus* were 16, 16, 32, and 64 μg/ml, respectively. For *E. coli*, the MBC and MIC values were identical, indicating that the effect of clove oil is mainly bactericidal and not bacteriostatic. This is due to the presence of eugenol as organic phenolic compound found in clove oil, which can denature proteins. Eugenol can react with phospholipids from cell membrane, and also it

Table 7
Desirability specifications for clove oil nanoemulsion

Name	Goal	Lower limit	Upper limit
Clove oil concentration (wt%)	within the range	2.5	15
Emulsifier concentration (wt%)	within the range	2.5	7.5
Mixed HLB number	within the range	9.0	15
Ultrasonication time (s)	within the range	150	450
Mean droplet size (nm)	is targeted 50	28.8 ± 1.6	310.3 ± 6.2
PDI	minimize	0.38 ± 0.06	0.56 ± 0.02
Zeta Potential (mV)	minimize	−40.1 ± 1.04	−10.4 ± 0.21

Table 8
Experimental and predicted optimal conditions for clove oil nanoemulsion

Factors							
A	*B*	*C*	*D*		Mean droplet size (nm)	PDI	Zeta potential (mV)
15	2.5	9	450	Predicted	47.75	0.509	−47.9
				Experimental	50.43 ± 0.91	0.496 ± 0.25	−40.7 ± 2.04

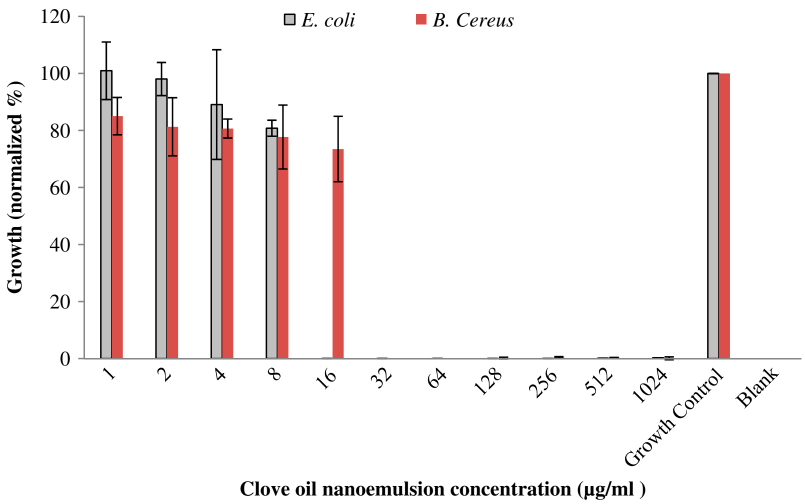

Fig. 5. Normalized growth vs. clove oil nanoemulsion concentration for MIC test.

interacts with cell membrane permeability in a great number of bacteria and yeasts [15,33–35]. The antibacterial activity of clove oil in comparison to clove oil nanoemulsion was insignificant due instability of raw clove oil. The MICs test for *E. coli* and *B. cereus* are shown in Fig. 5.

4. Conclusions

In this research, the processing factors for the preparation of clove nanoemulsion were optimized. The nanoemulsion is a biodegradable and green pesticide according to intended application. Based on Taguchi design method, the formulation of nanoemulsions consists of mixing three components, clove oil, water, and two different mixed surfactants via ultrasonication method. The error values according to Taguchi method for these experiments were insignificant. Hence, it was concluded that nearly all important and effective factors were considered and that errors in the experiments were also insignificant.

Based on targeted nanoemulsion with high degree of stability with zeta potential below −30 mV and lowest PDI obtained at optimum conditions a mean droplet size of 50 nm was achieved. The high degree of stability of nanoemulsion is secured by the value of zeta potential lower than −30 mV and low PDI value (sized within 50 nm).

The experiments for validation observed that mean droplet size, PDI, and zeta potential (32.2 ± 2.3 nm, 0.38 ± 0.06 and −40.7 ± 2.04 mV, respectively), were within the expected range of the value's for confidence limit, (30 nm, 0.374 and −47.9 mV, respectively).

To achieve all the properties at the same processing condition one has to go for a desired compromise by trading-off one of the nanopesticide properties. The optimum formula of clove oil nanoemulsion showed high antibacterial activity against all the tested bacteria. The present study suggests that the clove oil nanoemulsion is a potential source of natural antibacterial agents and to be used as food preservatives.

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References

[1] K.V. Plakas, A.J. Karabelas, Removal of pesticides from water by NF and RO membranes—A review, Desalination 287 (2012) 255–265.

[2] A. Biondi, N. Desneux, G. Siscaro, L. Zappalà, Using organic-certified rather than synthetic pesticides may not be safer for biological control agents: Selectivity and side effects of 14 pesticides on the predator *Orius laevigatus*, Chemosphere 87 (2012) 803–812.

[3] D.J. McClements, Edible nanoemulsions: Fabrication, properties, and functional performance, Soft Matter 7 (2011) 2297–2316.

[4] D.J. McClements, J. Rao, Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity, Crit. Rev. Food Sci. Nutr. 51 (2011) 285–330.

[5] H. Silva, M. Cerqueira, A. Vicente, Nanoemulsions for food applications: Development and characterization, Food Bioprocess Technol. 5 (2012) 854–867.

[6] I. Odriozola-Serrano, G. Oms Oliu, O. Martin-Belloso, Nanoemulsion-based delivery systems to improve functionality of lipophilic components, Front. Nutr. 1 (2014) 1–4.

[7] O. Sonneville-Aubrun, J.T. Simonnet, F. L'Alloret, Nanoemulsions: A new vehicle for skincare products, Adv. Colloid Interface Sci. 108–109 (2004) 145–149.

[8] M. Sivakumar, S.Y. Tang, K.W. Tan, Cavitation technology—A greener processing technique for the generation of pharmaceutical nanoemulsions, Ultrason. Sonochem. 21 (2014) 2069–2083.

[9] J. Gutierrez, C. Barry-Ryan, P. Bourke, The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients, Int. J. Food Microbiol. 124 (2008) 91–97.

[10] M. Hosseini, M.H. Shahavi, Electrostatic enhancement of coalescence of oil droplets (in nanometer scale) in water emulsion, Chin. J. Chem. Eng. 20 (2012) 654–658.

[11] J. Rao, D.J. McClements, Formation of flavor oil microemulsions, nanoemulsions and emulsions: Influence of composition and preparation method, J. Agric. Food Chem. 59 (2011) 5026–5035.

[12] L. Wang, X. Li, G. Zhang, J. Dong, J. Eastoe, Oil-in-water nanoemulsions for pesticide formulations, J. Colloid Interface Sci. 314 (2007) 230–235.

[13] F. Donsì, M. Annunziata, M. Sessa, G. Ferrari, Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods, LWT—Food Sci. Technol. 44 (2011) 1908–1914.

[14] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, Biological effects of essential oils—A review, Food Chem. Toxicol. 46 (2008) 446–475.

[15] M. Hyldgaard, T. Mygind, R.L. Meyer, Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components, Front. Microbiol. 3 (2012) 1–24.

[16] X. Kong, X. Liu, J. Li, Y. Yang, Advances in pharmacological research of eugenol, Curr. Opin. Complement Altern. Med. 1 (2014) 8–11.

[17] V. Ghosh, A. Mukherjee, N. Chandrasekaran, Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity, Ultrason. Sonochem. 20 (2013) 338–344.

[18] J.P. Canselier, H. Delmas, A.M. Wilhelm, B. Abismaïl, Ultrasound emulsification—An overview, J. Dispersion Sci. Technol. 23 (2002) 333–349.

[19] S.G. Gaikwad, A.B. Pandit, Ultrasound emulsification: Effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size, Ultrason. Sonochem. 15 (2008) 554–563.

[20] H. Majeed, J. Antoniou, C.F. Shoemaker, Z. Fang, Action mechanism of small and large molecule surfactant-based clove oil nanoemulsions against food-borne pathogens and real-time detection of their subpopulations, Arch. Microbiol. 197 (2015) 35–45.

[21] M. Ezoddin, J.Å. Jönsson, A. Kyani, Equilibrium sampling through membrane based on a hollow fiber for determination of naproxen and diclofenac in sludge slurry using Taguchi orthogonal array experimental design, Desalin. Water Treat. 52 (2014) 2472–2480.

[22] S.B. Otieno, C.M. Anderson-Cook, Design and analysis of experiments for directional data, in: K. Hinkelmann (Ed.), Design and Analysis of Experiments: Special Designs and Applications, John Wiley & Sons, Inc., Hoboken, New Jersey, 2012, pp. 501–532 (Chapter 15).

[23] Y. Li, Z. Teng, P. Chen, Y. Song, Y. Luo, Q. Wang, Enhancement of aqueous stability of allyl isothiocyanate using nanoemulsions prepared by an emulsion inversion point method, J. Colloid Interface Sci. 438 (2015) 130–137.

[24] N.G. Eskandar, S. Simovic, C.A. Prestidge, Interactions of hydrophilic silica nanoparticles and classical surfactants at non-polar oil–water interface, J. Colloid Interface Sci. 358 (2011) 217–225.

[25] S.S. Sagiri, B. Behera, T. Sudheep, K. Pal, Effect of composition on the properties of Tween-80–Span-80-based organogels, Des. Monomers Polym. 15 (2012) 253–273.

[26] E.S. Mahdi, M.H. Sattar, M.F. Sakeena, M. Abdulkarim, A.M. Noor, G. Abdullah, Effect of surfactant and surfactant blends on pseudoternary phase diagram behavior of newly synthesized palm kernel oil esters, Drug Des., Dev. Ther. 5 (2011) 311–323.

[27] G. Lv, F. Wang, W. Cai, X. Zhang, Characterization of the addition of lipophilic Span 80 to the hydrophilic Tween 80-stabilized emulsions, Colloids Surf., A 447 (2014) 8–13.

[28] M.J. Rosen, J.T. Kunjappu, Surfactants and Interfacial Phenomena, John Wiley & Sons Inc, Hoboken, New Jersey, 2012, pp. 336–367.

[29] F. Deniz, Optimization of methyl orange bioremoval by *Prunus amygdalus* L. (almond) shell waste: Taguchi methodology approach and biosorption system design, Desalin. Water Treat. 51 (2013) 7067–7073.

[30] A. Reyhani, F. Rekabdar, M. Hemmati, A.A. Safe-Kordi, M. Ahmadi, Optimization of conditions in ultrafiltration treatment of produced water by polymeric membrane using Taguchi approach, Desalin. Water Treat. 51 (2013) 7499–7508.

[31] A. Salahi, T. Mohammadi, M. Nikbakht, M. Golshenas, I. Noshadi, Purification of biologically treated Tehran refinery oily wastewater using reverse osmosis, Desalin. Water Treat. 48 (2012) 27–37.

[32] A. Kumar, B. Bhunia, D. Dasgupta, T. Mandal, A. Dey, S. Datta, P. Bhattacharya, Optimization of culture condition for growth and phenol degradation by *Alcaligenes faecalis* JF339228 using Taguchi Methodology, Desalin. Water Treat. 51 (2013) 3153–3163.

[33] M. Okshevsky, R.L. Meyer, Evaluation of fluorescent stains for visualizing extracellular DNA in biofilms, J. Microbiol. Methods 105 (2014) 102–104.

[34] M. Okshevsky, R.L. Meyer, The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms, Crit. Rev. Microbiol. 41 (2015) 1–11.

[35] M. Hyldgaard, D.S. Sutherland, M. Sundh, T. Mygind, R.L. Meyer, Antimicrobial mechanism of monocaprylate, Appl. Environ. Microbiol. 78 (2012) 2957–2965.