RESEARCH ARTICLE

Preparation and characterization of O/W nanoemulsion with *Mint* essential oil and *Parsley* aqueous extract and the presence effect of chitosan

Najmeh Feizi Langaroudi[1], Negar Motakef Kazemi[2,*]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Chemistry, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

[2] Department of Medical Nanotechnology, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

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ABSTRACT

Objective(s): The oil-in-water (O/W) nanoemulsion is expanded in biomedical application due to their special properties. *Mint* and *Parsley* are known herbs with many health benefits. Chitosan (Ch) is a low toxic, biodegradable, biocompatible and safe polymer with the antibacterial activity which is used in production of nanomaterial. The aim of this study was to evaluate the nanoemulsion to achieve good antibacterial activity and suitable stability with low cytotoxicity.

Methods: In the presence study, the O/W nanoemulsion was prepared by *Mint* essential oil in the presence of *Parsley* aqueous extract and chitosan aqueous solution using Span 80 and Tween 80 surfactants and high-intensity homogenizer at room temperature for 30 min. The result samples were characterized by scanning electron microscopy (SEM), and dynamic light scattering (DLS). The stability of nanoemulsion was evaluated for 50 days at different temperatures for optimized samples. The antibacterial activity of samples was measured against an important pathogen bacterium, *Escherichia coli* (*E. coli*) using inhibition zone diameter. The rheological properties of nanoemulsion were determined by presentation of viscosity at room temperature. The cytotoxicity of samples was investigated by MTT assay for HEK239 human cell line at three different concentrations for three periods of time.

Results: Our results showed the nanoemulsion with nanometer size. The presence of chitosan was caused more particles with the smaller size. The low temperature was needed to protection of nanoemulsion stability and prevention of particle growth.

Conclusions: Our study indicated that the nanoemulsion improved its antibacterial activity with low cytotoxicity and suitable stability.

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INTRODUCTION

Nanotechnology has been led to a significant progress on various scientific fields and product innovation. Over the past years, microemulsions have attracted much interest as delivery systems because of unique properties such as ease of in preparation, transparency, and long-term stability [1]. The nanoemulsions have been extensively studied because of their potential applications in industrial various such as food, cosmetic, pharmacology, and agriculture in recent years [2,3]. Nanoemulsions are considered as intermediates between classical emulsions and microemulsions usually in the 20 to 500 nm size range [4-6]. The small size of nanoemulsion can be enhancing either the penetration into or absorption by cells [7]. The main categories of nanoemulsions are oil-in-water or water-in-oil, transparent or translucent, colloidal

* Corresponding Author Email: *motakef@iaups.ac.ir*

dispersions [8-10]. The components of oil-in-water emulsions can be included the lipophilic bioactive within the oil phase and an aqueous medium in presence of emulsifier. The produced droplet size depends on the component composition and the homogenization method. The thermodynamically unstable can be seen for oil-in-water conventional emulsions and nanoemulsions [11-14]. Oil-in-water nanoemulsions were stabilized by emulsifier such as Tween 20 as a non-ionic surfactant in aqueous buffer solution [15,16].

The nanoemulsions can be prepared with small particle size by high-energy or by low-energy emulsification methods and the surfactant is used to stability maintain. The high-pressure homogenizer is one of high-energy methods for the formation of nanoemulsions [17,18]. Nanomulsions have advantages than emulsion due to their small size, such as higher optical clarity, higher stability, and enhanced bioavailability of lipophilic compounds [19]. However, their Nanomulsion application is limited because of potential risks of nanostructures, such as the potential toxicity of some components (e.g. surfactants) and their ability to change the biological fate of materials and bioactive compounds, which could potentially have some adverse effects on human health [20,21].

The resistant bacteria have become a major challenge in developed countries [22,23]. The herbal oils as natural antimicrobial substances can be used as oil phase in emulsion systems to increase their efficacy and inhibit bacterial growth [24-26]. The antimicrobial activity of essential oils has been widely investigated in the last decades [27,28]. The herbal oil can be used as oil core in oil-in-water nanoemulsion [29]. The large number of volatile organic compounds is emitted from plants into the environment. These volatile compounds can be caused therapeutic and pharmacological properties [30,31].

The wide range of emulsions can be assembled with biomaterials. *Mint* is one of hundreds of different types of essential oils and the most popular [32]. *Mint* essential oils can be used as oil soluble part of emulsion. Recently, the antibacterial activities of *Mint* oil were evaluated against some Gram-positive and Gram-negative bacterial strains by agar well diffusion method [33-35]. The antioxidant properties of *Mint* oil were presented because of phenols one of the major groups of herbal natural compounds acting as radical scavengers that given the beneficial effect to human health [34-37]. Recently, *Parsley* extract was used for green synthesis of nanoparticles [38], that its aqueous solution can be used as water soluble part of emulsion. *Parsley* is few of the aromatic herbs with antibacterial activities against various bacterial strains [39-41]. Recently, the antioxidant capacity of *Parsley* as kind of herbs has attracted increasing interest compared with chemical antioxidants [42]. Chitosan is a biopolymer with biocompatibility, biodegradability, non-toxicity, and antibacterial properties [43,44]. Recently, the used of chitosan has expanded with essential oils because of the special properties of them both [45,46]. The aim of the present work was to form nanoemulsion of *Mint* essential oils in the presence of *Parsley* aqueous extract and chitosan aqueous solution and investigation of antibacterial activity against *E. coli* as Gram-negative bacterial model and cytotoxicity on HEK239 human cell line. This study provides important new information about nanoemulsions based on essential oils and aqueous extract.

MATERIALS AND METHODS

Materials

All chemicals used were analytical grade. Ultra-pure water was used for the preparation of all reagents solutions. Chitosan (medium molecular weight) was obtained from Sigma–Aldrich. Tween 80, Span 80, and glacial acetic acid were purchased from Merck Millipore (Darmstadt, Germany). *Mint* essential oil and *Parsley* aqueous extract bought from Adonis Gol Daro (Iran).

Characterization

Dynamic light scattering was reported the size and size distribution of nanoparticles (ZEN314, England). Scanning electron microscope was employed to observe the morphology and size (KYKY EM3200, China). The rheological properties were studied by investigating viscosity based on spindle speed for samples using Reologica InstrumentsAB (Lund, Sweden). The nanoemulsions were allowed to equilibrate for 72 h prior to evaluation of their rheological properties. The nanoemulsions was placed under minimum stress of 0.05 Pa and a maximum stress of 550 Pa and 600 Pa. Rheograms were produced automatically in triplicate that exerted gradually the increasing of shear stress to the specified maximum stress. The antibacterial activities were evaluated by disk diffusion method against *Escherichia coli* bacteria, ATCC 1399, that procured from Islamic Azad University. The cytotoxicity of samples was investigated by MTT assay for HEK239 human cell line at three different concentrations for 1, 3, and 5 days.

Table 1. The percentage of nanoemulsion component.

Sample	*Mint* essential oil (%)	*Parsley* aqueous extract (%)	Surfactant (%)
I	4	94	2 (Span 80)
II	4	94	2 (Tween 80)
III	2	96	2 (Span 80)
IV	2	96	2 (Tween 80)
V	4	95	1 (Span 80)
VI	4	95	1 (Tween 80)

Table 2. The mean particle diameter of nanoemulsion.

Sample	The mean particle diameter (nm)
I	67.3
II	89.7
III	84.8
IV	191
V	88.8
VI	176.2

Nanoemulsion preparation

The nanoemulsions were prepared from a mixture of *Mint* essential oil and *Parsley* aqueous extract by homogenizing at 18000 rpm for 30 min with different percentages in presence of Tween 80 and Span 80 surfactants (Table 1).

RESULTS AND DISCUSSION

DLS

The dynamic light scattering was determined the mean particle diameter and the particle size distribution of nanoemulsion. DLS results showed a single-peak with a narrow distribution with different size duo to the nanoemulsion components (Table 2).

Based on these results, the optimum percentage was observed for the nanoemulsion that included *Mint* essential oil (4% w/w), *Parsley* aqueous extract (94% w/w), and surfactant (2% w/w). The nanoemulsion in presence of chitosan was prepared with the optimum percentage of components by dissolving of chitosan powder (1% w/w) with 1% (v/v) acetic acid in *Parsley* aqueous extract. Fig. 1 was shown the DLS diagram of nanoemulsion (I) and (II) without and with chitosan in presence of two surfactants. The mean particle diameter was observed 78.1 nm in presence of Span 80 and 138 nm Tween 80 for nanoemulsion (I) and (II) respectively. Based on DLS result, the optimum nanoemulsion was nanoemulsion (I) in presence of chitosan and Span 80 surfactant.

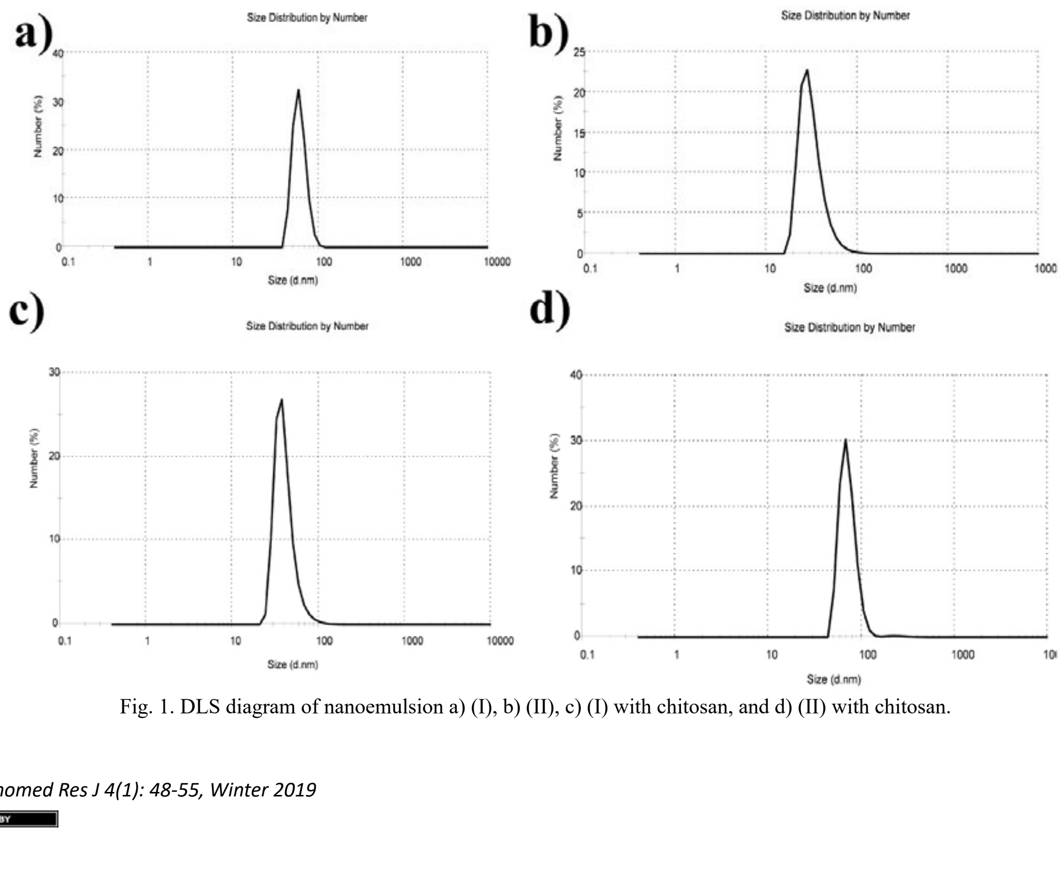

Fig. 1. DLS diagram of nanoemulsion a) (I), b) (II), c) (I) with chitosan, and d) (II) with chitosan.

The nanoemulsion has advantages than another oil-in-water nanoemulsions such as the smaller size compared with nanoemulsion of Sage oil with formulation containing sage oil, Tween 80 and Span 80 emulsifier, and water using probe sonicator in different times [47] and the narrow distribution compared with nanoemulsion of curcumin with formulation containing curcumin into the heated oil by homogenising [14]. The controlled size with the narrow distribution can be had potentially specific effects for different applications such as food and pharmaceutical industry in the future.

SEM

Fig. 2 was shown the SEM images for nanoemulsion (I) and (II) without and with chitosan in presence of Span 80 and Tween 80 with spherical morphology. The particle size was estimated for nanoemulsion (I) without and with chitosan about 70 nm (with broad distribution) and 60 nm (with narrow distribution) respectively. The particle size was estimated for nanoemulsion (II) without and with chitosan about 75 nm (with broad distribution) and 50 nm (with narrow distribution) respectively. According to the results, the presence of chitosan was caused more particles due to suitable nucleation and the smaller size because of the effect of capping agent. The advantages of the nanoemulsion include the spherical morphology with narrow distribution and more nanoparticles compared with previous report from researchers [47].

Rheological properties

The viscosity was investigated at different shear rates for nanoemulsions (I) and (II) without and with chitosan (Table 3). According to the result, the viscosity of sample was as following: nanoemulsions (I) with chitosan > nanoemulsions (II) with chitosan > nanoemulsions (I) >

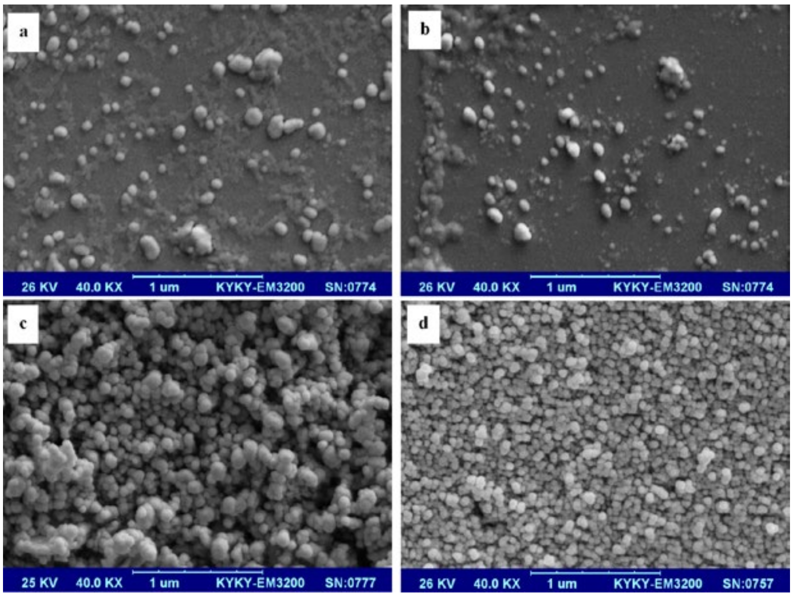

Fig. 2. SEM images of nanoemulsion a) (I), b) (II), c) (I) with chitosan, and d) (II) with chitosan.

Table 3. Rheological properties of nanoemulsions.

Sample	Spindle speed (round per minute)	Viscosity (centipoise)
Nanoemulsion with Span 80	60	6.5
Nanoemulsion with Tween 80	60	2
Nanoemulsion containing chitosan with Span 80	6	60
	12	57.5
	30	54
	60	51
Nanoemulsion containing chitosan with Tween 80	12	36
	30	35
	60	32.5

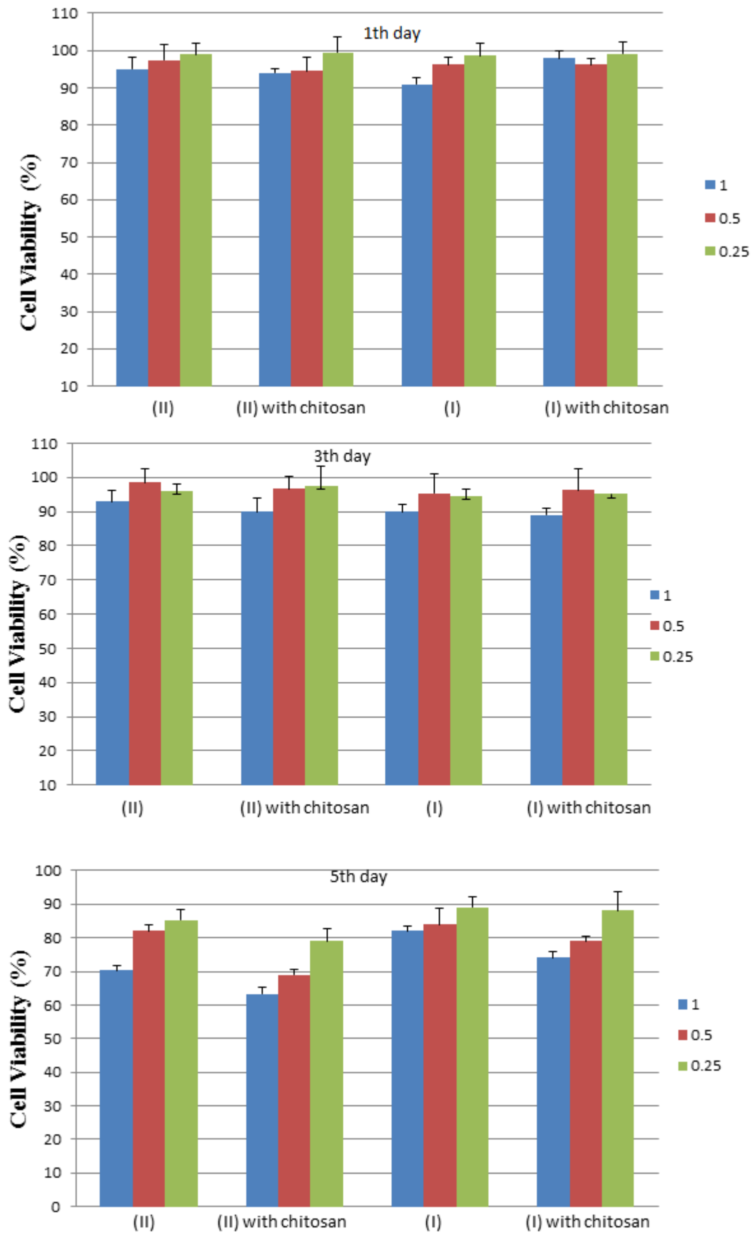

Fig. 3. The curves of nanoemulsion cytotoxicity in different times and concentrations.

nanoemulsions (II) in the same spindle speed. The higher viscosity was observed for nanoemulsions with chitosan in *Parsley* aqueous extract and Span 80 surfactant because of their nature and properties. The rheological properties are according to studies of Wulff-Pérez and coworkers in 2013 [49]. The flow ability of the nanoemulsion is determined by measuring spindle speed and viscosity, which indicated an important role in samples for food and pharmaceutical applications in future.

Antibacterial activity

The inhibition zone diameter was investigated for determination of sample antibacterial activity against *Escherichia coli* as Gram-negative bacterial model. The *E. coli* cells were cultivated on Mueller-Hinton Agar and incubated at 37 °C for 1 day. The inhibition zone diameter was 1 cm for nanoemulsions (I) and (II) and 1.5 cm for these nanoemulsions with chitosan. Based on the result, the presence of chitosan was increased antibacterial activity in nanoemulsions. The antibacterial

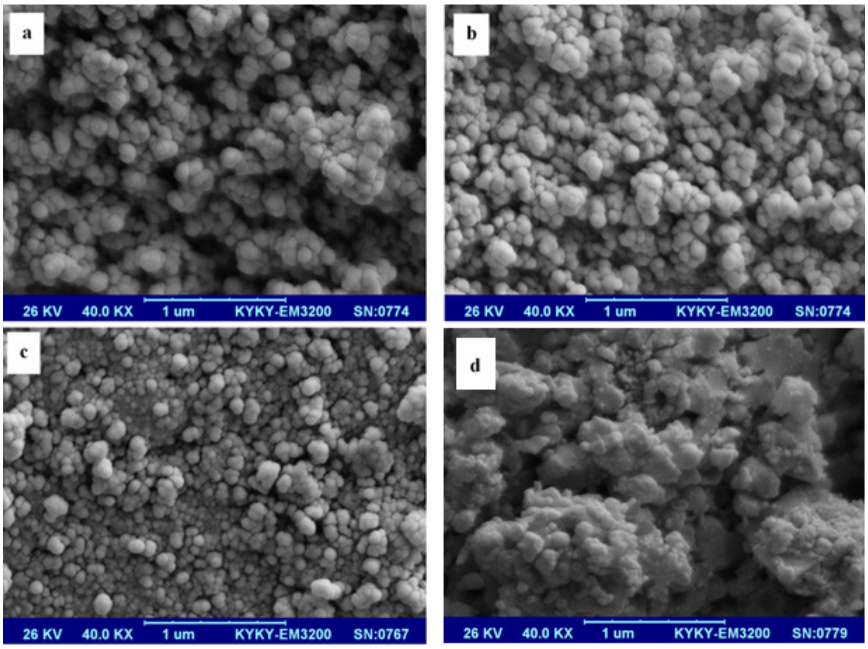

Fig. 4. SEM images of nanoemulsion (II) with chitosan after 5o days at the temperature of a) -20 Co, b) -4 Co, c) 15 Co, and d) 38 Co.

activity is according to previous reports from researchers [28, 47]. The antibacterial activity of nanoemulsion is related to essential oils and the presence of chitosan is caused the increase of antibacterial activity. The nanoemulsion can keep essential oils from easily evaporate and decompose during preparation, owing to direct exposure to heat, pressure and light [51].

MTT assay

The cytotoxicity of samples was investigated by MTT assay for HEK239 human cell line at three different concentrations 0.25, 0.5, and 1% (v/v) for three periods of time (1, 3 and 5 days). Based on the result, concentration and time were two effective factors on cytotoxicity of nanoemulsions. The higher cytotoxicity was observed because of the increase of concentration and time and the cell viability was shown more than 50% for all samples. The MTT assay advantages of the nanoemulsion include acceptable toxicity in longer time compared with previous report from researchers [50].

Stability

The stability was performed for the optimum sample that's mean nanoemulsion (I) with chitosan by SEM analysis into the different temperatures (-20, -4, 15, and 38 C^{o}) after 50 days. Based on the results, the particle size didn't change at -20, -4, and 15 °C, but the particle agglomerated at 38 °C (Fig. 4). Therefore, the low temperature was needed for protection of stable nanoemulsion to prevent growth and agglomeration of particle. The stability advantages of the nanoemulsion are according to studies of Mann and coworkers in 2015 and the increase of treatment caused to the increase of average size [48]. Nanoemulsion stability was reported for the first time in low temperature.

CONCLUSIONS

The present study demonstrated the nanoemulsion with *Mint* essential oil and *Parsley* aqueous extract in the presence of chitosan can be good candidate for antibacterial activity against *Escherichia coli* with low cytotoxicity for HEK239 human cell line. The ph ysical stability of nanoemulsion was good and suitable for 50 days at the temperatures of -20, -4, and 15 C^{o} with little evidence of phase separation or particle growth. The optimum sample was included *Mint* essential oil (4% w/w), *Parsley* aqueous extract (94% w/w), and Span 80 surfactant (2% w/w) in presence of chitosan. Based on our results, it can be proposed that the presence of chitosan can be controlled the size, nucleation, and antibacterial activity in the nanoemulsion. The prepared nanoemulsion can be a cost-effective way with good potential to different applications in variety research fields of food and pharmaceutical in future.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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