



Evaluation of Antioxidant Activity and Cytotoxicity of Cumin Seed Oil Nanoemulsion Stabilized by Sodium Caseinate- Guar Gum

Parastoo Farshi¹, Mahnaz Tabibiazar^{2*}, Marjan Ghorbani³, Hamed Hamishehkar³

¹Biotechnology Research Center and Student Research Committee, Department of Food Science and Technology, Faculty of Nutrition and Food Science, Tabriz University of Medical Sciences, Tabriz, Iran.

²Nutrition Research Center and Department of Food Science and Technology, Faculty of Nutrition and Food Science, Tabriz University of Medical Sciences, Tabriz, Iran.

³Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Article Info

Article History:

Received: 18 July 2017

Accepted: 17 October 2017

ePublished: 30 December 2017

Keywords:

-Cumin seed oil
-Nanoemulsion
-Sodium caseinate
-Guar gum
-Antioxidant activity
-Cytotoxicity

ABSTRACT

Background: The objective of this study was to prepare the sodium caseinate-guar gum stabilized nanoemulsion of cumin seed oil (*Cuminum cyminum*) using ultrasonication method. Meanwhile, the effect of nanoemulsification on the antioxidant and cytotoxicity of the cumin seed oil was evaluated.

Method: The effect of concentration of sodium caseinate and guar gum was investigated on droplet size, thermal and oxidative stability of cumin seed oil nanoemulsion using TBARS and z-average measurements, the antioxidant activity was evaluated by DPPH scavenging and iron reducing power measurements. The biocompatibility and the cytotoxicity of the cumin seed oil nanoemulsion were evaluated by MTT assay test and compared with cumin seed oil and cumin seed oil free-nanoemulsion.

Results: GC-MS analysis indicated 15 compounds in the cumin seed oil. The nanoemulsions were stabilized by sodium caseinate-guar gum complex. The minimum and stable droplets (155 ± 8 nm) of nanoemulsion were formulated when the concentration of essential oil in oil phase was 30 % (w/w). DPPH radical scavenging ability, iron reducing power and cytotoxicity of nanoemulsified cumin seed oil were significantly higher than cumin seed oil ($p < 0.05$)

Conclusion: In this study, cumin seed oil nanoemulsion was prepared and stabilized by sodium caseinate- guar gum. The aforementioned nanoemulsion had good stability even after 60 days storage at 4°C. Antioxidant and cytotoxicity of cumin seed oil were increased by nanoemulsification. It can be concluded that cumin seed oil nanoemulsion has the potential to use as natural preservative and anticancer product in food industry.

Introduction

Cumin seed oil (CSO) is an essential oil that is reported to be a rich source of bioactive compounds which are responsible for its antimicrobial activity.¹ In spite of good functional properties of essential oils, their application in food matrix is limited because of their hydrophobic and volatile nature.^{2,3} Nanoemulsions are systems with droplet diameters smaller than 200 nm⁴ that can stabilize oil droplets against instability of gravitational force and droplet aggregation.⁵ Recently antimicrobial O/W nanoemulsions have attracted a great interest in food industry because of their improved functionality. Sodium caseinate (SC) is a protein that is prepared from casein micelles. It is a well-used ingredient because of its good solubility and emulsifying

properties and its stability during processing.⁶ SC is composed of a mixture of four phosphoproteins: as₁, as₂, b- and k-casein.^{7,8} The general structure of SC is disordered. These properties enhance its affinity to adsorb onto the interface during emulsification thereby establishing a steric layer that protects droplets against flocculation and coalescence.⁹ Nevertheless, one of the drawbacks of the emulsions stabilized by SC is that they are extremely sensitive to destabilization in acidic conditions.¹⁰ Polysaccharides as stabilizing agents are capable of ameliorating this drawback by formation of stabilizing layer around the protein coated droplets.¹¹ Galactomannans are the most widely used polysaccharides in food industry as thickening agents. They have a linear backbone of (1±4) linked

*Corresponding Author: Mahnaz Tabibiazar, E-mail: mahnaz_tabibiazar@yahoo.com

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d-mannose residues substituted with side chains constituted by single (1±6)-d galactose residues.¹² Guar gum, which is a neutral galactomannan has a mannose to galactose ratio of about 2:1. This galactomannan is widely used in food industry as stabilizing and thickening agent, because of its good water holding properties. In this study we tend to produce CSO nanoemulsion stabilized with SC and guar gum and evaluate its antioxidant activity and cytotoxicity.

Materials and Methods

Materials

SC and CSO were obtained from Sigma-Aldrich Chemical Company, USA, and stored in the refrigerator at 4°C. Commercially available corn oil was purchased from the local supermarket and used as oil phase. 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide and trichloroacetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin-streptomycin solution (10000 units/mL of penicillin and 10 mg/mL of streptomycin), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Human breast epithelial adenocarcinoma MCF-7 cell line was supplied from NCBI (National Cell Bank of Iran, Pasteur Institute). RPMI 1640 and fetal bovine serum (FBS) (heat-inactivated) were purchased from GIBCO Invitrogen GmbH (Germany).

Nanoemulsion preparation

The aqueous phases of emulsions were prepared in three different concentrations of proteins (3, 5 and 10 % w/v). SC solution was prepared by dissolving its powder into double distilled water and then stirred to enable complete hydration. Oil composition (Corn oil / CSO) was varied to obtain a suitable ratio in order to prepare stable nanoemulsions with smaller droplet diameter. The ratio of oil phase (Corn oil + CSO) to aqueous phase was set at 10:90. A coarse emulsion was prepared by homogenization using high-speed Ultra-Turrax blender (Hiedoloph, Germany) at 22,000 rpm for 5 min. The coarse emulsion was further emulsified using 20 KHz ultrasonicator, UP 200S (Dr. Hielscher, Germany) with a maximum power output of 200 W. The amplitude of oscillation was set at 70 microns and ultrasonication was applied in 1 min intervals on emulsion in order to avoid any destructive effect on protein. The time of ultrasonication was set based on reaching nano size in droplets in order to avoiding the excess process. The temperature difference between the primary coarse emulsion and the final emulsion was less than 10°C. Increase in temperature during ultrasonication was inhibited by placing the sample container in a bigger beaker containing ice. The volume of the coarse emulsion was set to 5ml in all samples and

the sonotrode was located 1 cm below the surface of the emulsion. In order to evaluate the GG effect on SC-stabilized nanoemulsion different concentrations of GG (0.1, 0.2, 0.3 and 0.4) were added to the aqueous phase. The most suitable concentration of GG was selected based on droplet size and stability of the nanoemulsion.

Particle size and Zeta potential measurements

The particle size, polydispersity index (PDI) and zeta potential measurements were performed using a zeta sizer Nano ZS model ZEN 3600 (Malvern Instruments, UK). Particle size (hydrodynamic radius, Rh) measurements were done based on dynamic light scattering. The instrument determines the particle size distribution by measuring intensity fluctuations over time of a laser beam (633 nm) scattered by the samples at an angle of 173°. Zeta potential measurements were performed based on laser Doppler anemometry, using the same machine. For zeta potential measurements, Samples were loaded into pre-rinsed folded capillary cells and a minimum of three measurements were made per sample. Prior to any measurements being taken, the samples were diluted with 250-fold bi-distilled water to reach a suitable concentration. Physical stability of emulsions was studied by droplet size measurement in one month interval during two months.

Viscosity measurements

Viscosity measurements of oil and aqueous phases were performed by Physica MCR 301 Rheometer® (Anton Parr, Austria) using double-gap concentric cylinder geometry and ramping shear rate profile from 0.1 to 1000 s⁻¹ at 25°C.

Interfacial tensions measurements

Interfacial tensions between the aqueous phase and oil phase (Corn oil+ CSO) were measured using the DU Nouy ring method with a tensiometer (WHITE®-England) at 25 °C. Triplicate tests were performed for each measurement.

Scanning electron microscope analysis

The morphology and structure of protein stabilized nanoemulsions were visualized using scanning electron microscope (SEM). To perform scanning electron microscopy, a nanoemulsion was diluted 250 folds in distilled water and mounted on lamels until dried at room temperature. Samples were observed under a high resolution KYKY-EM 3200microscope (Beijing, China). Photographs were taken at excitation voltage of 26 KV.

DPPH scavenging activity measurements

The 2,2-diphenyl-1-picrylhydrazil (DPPH) scavenging activities of the samples were measured, using the method described by Brand-Williams et al,

1995 with minor modifications.¹³ Briefly, 1 ml of pure CSO (400 µg/ml) or CSO nanoemulsion (400 µg/ml) was mixed with 2 ml of ethanolic DPPH solution (0.125 mM). Mixtures were incubated for 1 h at 25 °C in the dark and then centrifuged at 15,000 rpm for 30 min. The supernatant was taken and the absorbance was measured at 517 nm, using a Nicolet evolution 300 UV-Vis spectrophotometer (Thermo electron corporation, England). Blank samples of free pure CSO and the nanoemulsion were prepared, respectively, by mixing 1 ml of double distilled water or nanoemulsion without CSO with 2 ml of DPPH- ethanolic solution. The percentage of DPPH scavenging activity of the samples was calculated as following:

$$I\% = 1 - [A_{\text{sample}} / A_{\text{blank}}] \times 100 \quad \text{Eq.(1)}$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50 % inhibition (IC50) was calculated from the plot of inhibition percentage against extract concentration. Tests were carried out in triplicate.

Iron reducing power

The capacity of essential oils to reduce Fe^{3+} was assessed according to a reported method by Dinis, et al 1994 with modification.¹⁴ The essential oils were mixed with 2.5 ml of PBS buffer (pH 6.5, 0.2 M) and 2.5 ml of potassium ferricyanide (1%). This mixture was incubated at 50 °C for 20 min. After the addition of trichloroacetic acid (2.5 ml, 10%), the new mixture was centrifuged for 10 min at 650×g. Then, the upper layer (2.5 ml) was mixed with deionized water (2.5 ml) and ferric chloride (0.5 ml). The absorbance was measured at 700 nm. A higher absorbance indicates a higher reducing power. The IC50 value (µg/mL) is the extract concentration at which the absorbance was 0.5 for the reducing power and was calculated from the graph of absorbance at 700 nm against extract concentration.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed on a Hewlett-Packard 5973 system with HP 5MS column (30 m × 0.25 mm, film thickness 0.25 µm). The column temperature was kept at 60 °C or 3 min and programmed to reach 220°C at a rate of 5°C/min and stayed steady at 220 °C for 3 min. The components of the oil were then identified by comparison of their mass spectra and retention indices (RI) with those given in literature and those of the authentic samples.

Cell cytotoxicity study

The biocompatibility and the cytotoxicity of the CSO nanoemulsion were evaluated by MTT assay test against MCF-7 cell line and compared with CSO

and CSO free-nanoemulsion as positive and negative controls. The cells were seeded into a 96-well plate at a density of 1×10^4 cells per well. After incubation for 24 h (37°C, 5 % CO_2), the culture medium was removed and 200 mL of growth medium containing various concentrations of free CSO and CSO nanoemulsion was added to each well and incubated for 24 h, 48 h and 72 h. After finishing, the incubated medium was taken out and the MTT solution ($5 \text{ mg} \cdot \text{mL}^{-1}$) was added and cells were incubated for 4 h. The medium was aspirated, the MTT-formazan was dissolved in 200 µL of DMSO, and the optical density (OD) was measured at 570 nm by using a microplate reader (Elx808, Biotek, USA) and the results were compared with respect to control cells.

Statistical analysis

All experiments were carried out triple. One-way analysis of variance was performed using SPSS software v18 to assess the statistical significance of difference within the samples. Results with ($p < 0.05$) were considered statistically significant. Duncan's multiple range tests were used to compare treatment means in triplicate.

Result and Discussion

GC- MS analysis

GC-MS analysis showed that CSO is mainly consisted of gamma-Terpinene (16.89 %), Pulegone (42.55), Beta pinene (8.6 %), Beta myrcene (0.64 %), P-cymene (11.76 %), D-Limonene (1.27 %), and Alpha-Thujenal (7.94%). Total GC-MS results are shown in Table 1.

Table 1. Cumin seed oil composition through GC-MS.

	Compound name	Retention index ^a	%Area
1	Ethyl Acetate	612	1.41
2	Alpha.-Pinene	934	2.07
3	2-β-pinene	978	8.66
4	β-Myrcene	989	0.64
5	P- cymene	1010	11.76
6	D-Limonene	1022	1.27
7	γ-Terpinene	1054	16.89
8	α.-Thujenal	1178	7.94
9	Pulegone	1201	0.83
10	α-Terpineol	1209	0.35
11	Cuminaldehyde	1230	42.55
12	Phellandral	1263	7.94
13	Carvacrol	1300	0.26
14	Cuminic acid	1331	0.45
15	1-Phenyl-1-propanol	1560	3.00

^a The retention Kovats indices were determined on HP-5 capillary column.

Effect of protein concentration and guar gum on droplet diameter

In order to evaluate protein concentration effect on

droplet diameter, 3, 5 and 10 % SC concentrations were selected to produce nanoemulsions. Results showed that increasing SC concentration up to 5 wt. % has significant effect ($p < 0.05$) on decreasing droplet diameter (227 ± 8 nm), but further increase in protein concentration (up to 10 wt. %), didn't have any significant effect on droplet diameter (255 ± 11 nm) (Figure 1). Nanoemulsions with 10 wt. % SC were not stable and creaming occurred after 6 days storage at 4°C . However, when the protein surface concentration is low (3 wt. %), bridging flocculation occurs that causes instability. By increasing protein content (3 to 5 wt. %), there are sufficient protein content to provide acceptable surface coverage (Depletion flocculation was minimized) and thus, provide good stabilization. These results are in agreement with the study done by Huck-Iriart *et al* 2011 that has indicated that a concentration of 5 wt. % NaCas produces an emulsion with a very homogeneous droplet distribution and a lower droplet diameter (281 nm). Due to a higher concentration of NaCas in the aqueous phase this emulsion apparently had more protein for the coverage of oil droplet surface.⁶ Further increasing of protein concentration up to 10 wt.% causes bridging flocculation due to non-adsorbed proteins on the surface.¹⁵

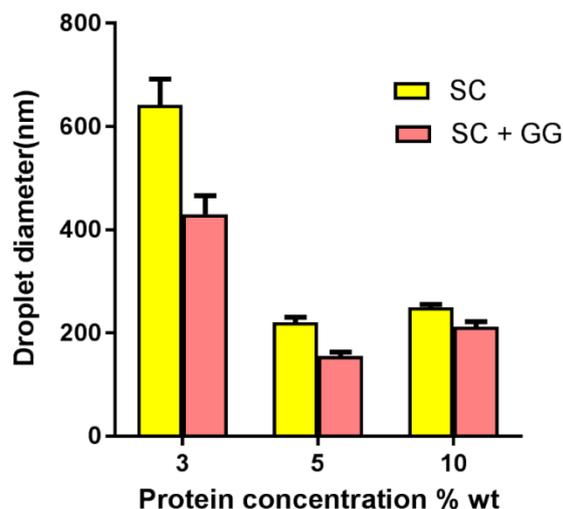


Figure 1. Effect of Sodium caeinate concentration (2, 5 and 10 % wt) and Sodium caseinate – Guar gum concentration (0.2 % wt) on droplet diameter of cumin seed/corn oil in water 10: 90 emulsion.

Our results were in agreement with other studies that they have found that there is very low creaming stability in emulsions made with high SC concentration (more than 8 wt. %), because of non-adsorbed proteins on the surface and depletion flocculation.¹⁶ In order to produce stable SC nanoemulsions, in the broader pH range, effect of GG as nonionic polysaccharide on droplet diameter was evaluated. Different amounts of GG (0.1, 0.2, 0.3, 0.4 wt. %) were added to the nanoemulsion. By

addition of GG up to 0.2 wt. % the droplet diameter of the nanoemulsion was decreased but further increase in concentrations of GG had not significant effect on decreasing droplet diameter. As shown as in Figure 2 the nanoemulsion contained 0.2 wt. % GG was more stable even after 30 days of storage time. By addition of GG, the droplet diameter of the SC (5 wt. %) nanoemulsion was decreased to 155 ± 8 nm.

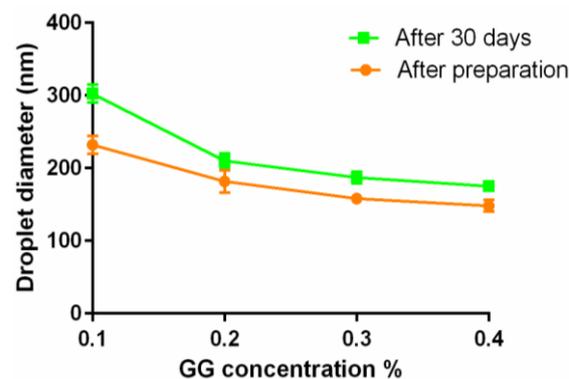


Figure 2. Droplet diameter changes of Sodium caeinate stabilized O/W (10: 90) nanoemulsion in the presence of different concentrations of guar gum.

GG has a positive role on the stability of SC-stabilized nanoemulsions and its effect depends on its concentration. This polysaccharide has some effects such as improving the SC adsorption at the oil–water interface, the prevention of re-coalescence of oil droplets, as well as the diminishing of average droplet diameter and partial coalescence of fat, to name but few.^{17,18} Zeta potential of SC and SC-GG nanoemulsions were -34.4 ± 0.2 and -40 ± 0.3 mV respectively. It may suggest that the electrostatic repulsion is the main factor preventing particle aggregation. Moreover, smaller droplet diameter and thicker interface of SC and GG blend caused to be more stable than SC stabilized nanoemulsions. Figure 3 shows SEM results of SC-GG and SC stabilized nanoemulsions. Besides, the droplet diameter changes of the nanoemulsion after storage was highly dependent on the ratio of CSO to corn oil in the lipid phase. Corn oil acts as an Ostwald ripening inhibitor.¹⁹ By increasing CSO level (>50% in lipid phase) the emulsions were unstable and consequently visible phase separation and droplet growth occurred, but at lower levels (30%) the droplet size was relatively low (227 ± 8 nm) (Figure 4).

Interfacial tension and viscosity measurements

Dispersed to continuous phase viscosity ratio and interfacial tension are the two parameters that must be engineered in nanoemulsions' production.²⁰ The optimum (η_D/η_C) of nanoemulsions to produce nanoparticles is 0.1- 5.²¹

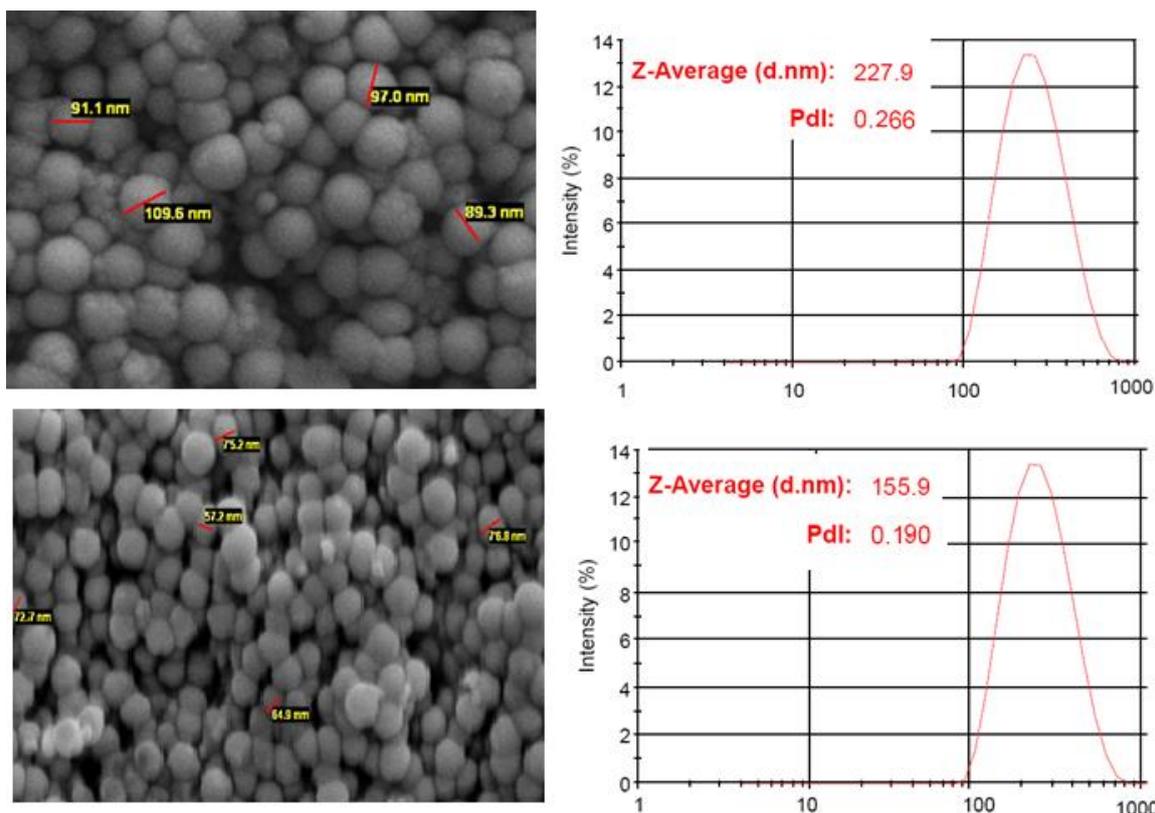


Figure 3. The SEM micrograph and Z- average curves of nanoemulsions Stabilized with :Sodium caeinate 5 % wt (A), Sodium caseinate 5 %– Guar gum 0.2 % (B) (pH=6.7 ± 0.1).

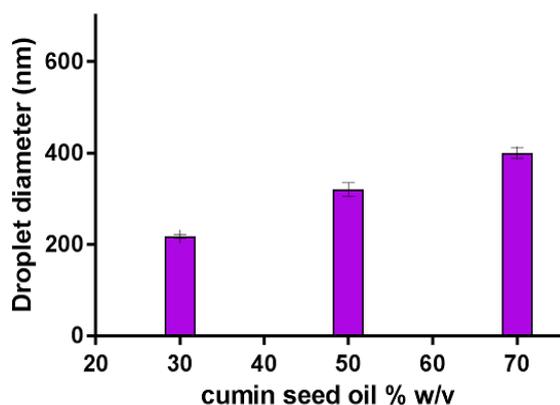


Figure 4. Changes in droplet diameter of O/W (10: 90) nanoemulsion by replacement of different concentration of CSO.

Results from viscosity measurements (Figure 5) showed that GG leads to increase in apparent viscosity of SC solution and when the dispersed to continuous-phase viscosity ratio (η_D/η_C) is in the range of 0.1- 5, which decreases the cavitation's threshold and consequently decreases the droplet diameter. Viscosity measurement results showed that the casein 5 % suspension was viscous to some extent (5.24 mPa.s at 1000 s⁻¹) and exhibited a Newtonian behavior. The 0.2 % GG solution had shearthinning behaviour with an apparent viscosity of 6 mPa.s at 1000 s⁻¹ shear rate.

With GG addition the viscosity of the mixture

increased to 12.9 mPa.s. Our results showed that there is an interaction between two biopolymers that causes to increase in apparent viscosity. Interfacial measurements showed that SC is able to decrease interfacial tension of water from 36 to 9.5 mNm⁻¹. Addition of GG didn't have any significant effect on decreasing interfacial tension of aqueous phase of SC stabilized nanoemulsions because GG is not a surface active polysaccharide.

Stability of the nanoemulsions

In order to evaluate the stability of the nanoemulsions they were held in 4 °C for 60 days. The droplet size, zeta potential and PDI measurements were done immediately after preparation and after 60 days of storage in the refrigerator (4 °C). Results showed that the nanoemulsion stabilized by SC-GG was more stable during this period of time that proves the effect of GG on diminishing the droplet diameter and boosting the stability of the whole system as well (Table 2).

Antioxidant activity of pure and nanoemulsified CSO

Plant phenols and flavonoids are capable of inhibiting lipid peroxidation by quenching lipid peroxy radicals and chelate iron through lipoxxygenase enzyme and thus preventing beginning of lipid peroxidation reaction.²²

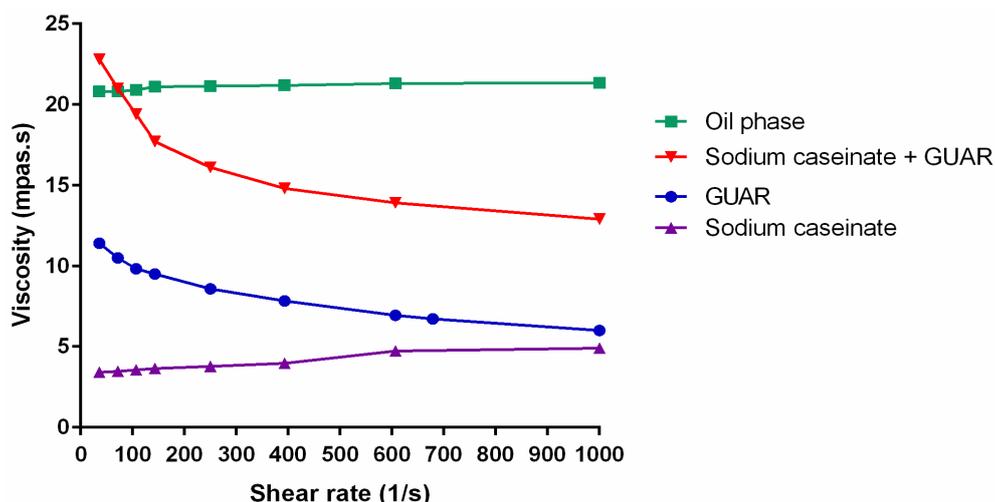


Figure 5. Apparent viscosity of oil phase (30:70 cumin seed: corn oil) and aqueous phases including Sodium caseinate 5 %–Guar gum 0.2 % wt, Guar gum 0.2 % wt, and Sodium caseinate 5 % wt.

Table 2. Zeta potential, Droplet diameter and Poly dispersity index (PDI) of the nanoemulsions immediately after preparation and after 30 days of storage at 4°C.

Nanoemulsions	Zeta potential		Droplet diameter		PDI	
	1 days	30 days	1 day	30 day	1 day	30 day
SC	^a -34 ± 0.4	^c -25 ± 0.3	^e 227 ± 8	^g 243 ± 11	ⁱ 0.276 ± 0.034	^j 0.411 ± 0.054
SC-GG	^b -40 ± 0.3	^d -33 ± 0.2	^f 155 ± 8	^h 185 ± 10	ⁱ 0.145 ± 0.065	^k 0.294 ± 0.034

The superscripts a-k indicate the significance of the mean difference in each column ($p < 0.05$).

At a concentration of 265 ± 0.3 $\mu\text{g/ml}$, the iron reducing power of nanoemulsified essential oil was 50 %, while IC_{50} of pure essential oil was 310 ± 0.5 $\mu\text{g/ml}$. There was a significant differences ($p < 0.05$) between these two measurements.

DPPH -scavenging activity of the samples was the second method in order to evaluate antioxidant activity. 4.5 ± 0.02 $\mu\text{g/mL}$ of nanoemulsified CSO has scavenging ability of 50 % that was significantly higher ($p < 0.05$) than the pure essential oil (27.5%). 5.8 ± 0.03 mg/mL of pure CSO is able to scavenge 50 % of free radicals (Table 3). By increasing the essential oil's concentration to 0.1 $\mu\text{g/mL}$, radical scavenging ability of nanoemulsified and pure essential oil increased to 39.3% and 73.4% respectively. CSO presents a strong antioxidant activity, which is in agreement with the previous literatures. This is due to powerful inhibitor compounds, which may act as primary antioxidants that react with free radicals as efficient hydrogen donors.²³

In vitro cell cytotoxicity and biocompatibility

In order to assess the cytotoxic effects of CSO, its nanoemulsion and the nanoemulsion without CSO at concentrations ranging from 0.01 to 1000 $\mu\text{g.mL}^{-1}$, they were treated with MCF-7 cells for 24 h, 48 h and 72 h. As shown as in Figure 6, the cell viability of nanoemulsion without CSO displayed no significant toxicity on MCF-7 cell's viability even after 72 h, approving that the free CSO-nanoemulsion revealed good biocompatibility of the prepared nanoparticles. The cytotoxicity profiles of the CSO nanoemulsion showed the same or even slightly higher inhibition activity after 24 h, 48 h and 72 h in comparison with pure CSO which would be valued in "Enhanced Permeability Retention" effect of the nanoemulsion and accumulation in cancerous tissues by passive targeting of CSO nanoemulsion. This is more dominant in the cases like CSO which shows cytotoxic effect in high concentrations. The results showed that the nanoemulsion of CSO offers an efficient anticancer delivery system and has much favorable potential as drug carriers. The blank nanoemulsion (without CSO) didn't have significant effect on cancer cells.

Table 3. IC_{50} of DPPH and Iron reducing power for pure and nanoemulsified cumin seed oil.

	DPPH, IC_{50} ($\mu\text{g/mL}$)	Chelating ability Reducing power IC_{50} , ($\mu\text{g/mL}$)
Cumin seed oil	^a 5.8 ± 0.03	^b 310 ± 0.5
Nanoemulsified	^a 4.5 ± 0.02	^c 265 ± 0.3

The superscripts a-c indicate the significance of the mean difference in each column ($p < 0.05$).

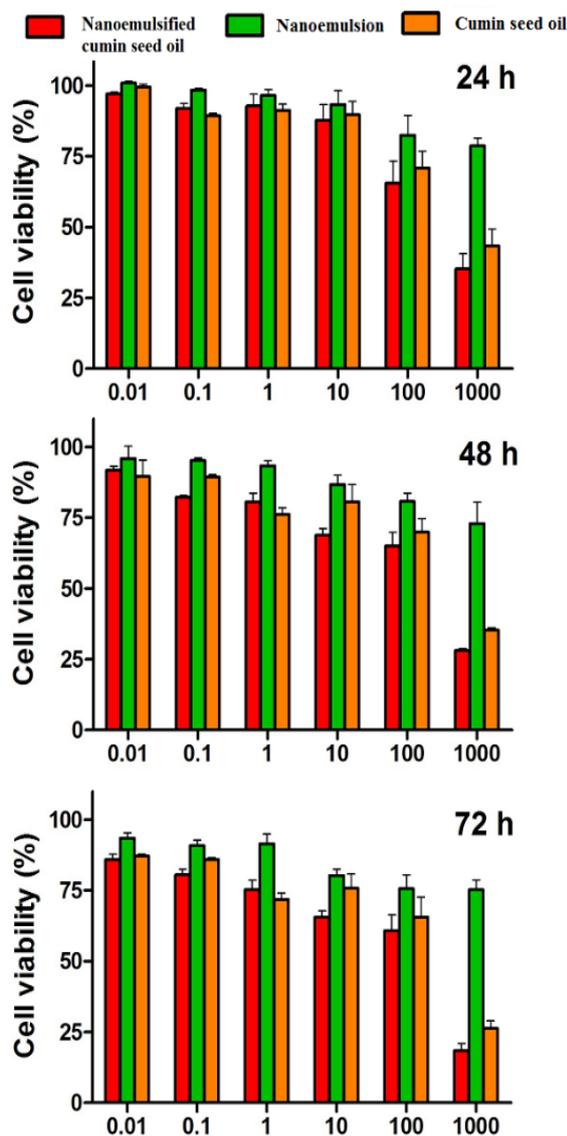


Figure 6. The Cell viability of the nanoemulsified CSO, nanoemulsion (blank, without CSO) and CSO against MCF-7 cell line (Human breast epithelial adenocarcinoma) for 24 h (a), 48 h (b) and 72 h (c). (Data are presented as mean \pm standard deviation, n = 6).

Conclusion

Through GC-MS analysis, 15 compounds were identified in the CSO. After optimization of oil phase composition, our studies demonstrated that sodium caseinate - guar gum is suitable formulation for CSO nanoemulsion. The nanoemulsification significantly increased the antioxidant activity of the essential oil. The results showed that the nanoemulsion of CSO can be used as efficient anticancer delivery system and has much favorable potential as drug carriers. Therefore, nanoemulsification provides an excellent way to improve the activity and efficiency of oil-soluble active agents significantly, and greatly expands their application in various aqueous foods.

Acknowledgment

The research vice-chancellor of Tabriz University of Medical Sciences financially supported this study.

Conflict of interests

The authors claim that there is no conflict of interest.

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