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Preparation of O/W nano-emulsion containing nettle and fenugreek extract and cumin essential oil for evaluating antidiabetic properties

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Abstract

The oil-in-water (O/W) nano-emulsion (NE) is expanded to enhance the bioavailability of hydrophobic compounds. The NE can be prepared by herbal extract and essential oil as herbal medicines for antidiabetic treatment. In the present study, the O/W NE was prepared by fenugreek extract (FE), nettle extract (NE), and cumin essential oil (CEO) using tween 80 and span 80 surfactants in an ultrasonic bath, at room temperature within 18 min. The antidiabetic property was evaluated by determining glucose absorption using cultured rat L6 myoblast cell line (L6) myotubes and insulin secretion using the cultured mouse pancreatic beta-cell (RIN-5) for NEs. The samples were investigated by dynamic light scattering (DLS) to examine the size distribution and size, zeta potential for the charge determination, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) to investigate morphology and size. The rheological properties were studied by viscosity. The sample stability was evaluated at different temperatures and days by DLS and SEM analyses. The cytotoxicity of samples was explored by MTT assay for HEK293 human cell line as a specific cell line originally derived from human embryonic kidney cells at three different concentrations for three periods of time. The NEs with nanometer-size were observed with antidiabetic properties, low cytotoxicity, and suitable stability. This study provides definitive evidence for the NE as a plant medicine with antidiabetic properties. The NE can be a good candidate for biomedical applications.

Keywords: Nano-emulsion, Aqueous extract, Nettle, Fenugreek, Cumin essential oil

Introduction

Diabetes refers to a rise in glucous blood levels because of insulin deficiency or malfunction (Modak et al., 2007). Diabetes is the fifth leading cause of death worldwide (Kazi, 2014). Nowadays, diabetes disease is on the rise globally (Mukesh & Namita, 2013). Uncontrolled diabetes will cause an increase in the risk of some other diseases; therefore, it is vital to control and treat it (Laligani

et al., 2005). Diabetes has two forms called type 1 diabetes mellitus (DM) and types 2 DM (Landon et al., 2009). There are various treatments such as medication, insulin, and diet to control diabetes (Bathaie et al., 2012); however, there are disadvantages, including side effects and drug resistance in these treatments (Dey et al., 2002).

Nanotechnology has developed in different fields because of its extensive practical benefits (Ataei et al., 2020; Motakef Kazemi & Sandalnia, 2020). Nanomaterials have potential uses due to their small size and high surface area (Jeevanandam et al., 2018; Motakef Kazemi et al., 2020). Over the past years, emulsions and nano-emulsions (NE) have attracted much interest as drug delivery systems and gene therapy (Zeinali et al., 2020)

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because of their transparency, ease of preparation, and long-term stability (Pickup et al., 2008). The emulsion method was used for preparing lipid-based nanoparticles (NPs) such as solid lipid nanoparticles (SLNs), micelle such as normal micelle, polymeric NPs, and inorganic/metallic NPs (Zeinali et al., 2020). NEs can incorporate a high lipid with more homogeneous nanoparticle distributions. At the same time, SLNs can have a lower total lipid that would lead to the formation of nanoparticles with lower homogeneous particle distribution (Deli et al., 2009). The main types of NEs are oil-in-water (O/W), water-in-oil (W/O), water in oil in water (W/O/W), and oil in water in oil (O/W/O) (Hormann & Zimmer, 2016). Recently, O/W NE was evaluated based on the aqueous extract and essential oil for biological applications (Feizi Langaroudi & Motakef, 2019). NE technology is particularly suitable for encapsulating active compounds, preventing their degradation and improving their bioavailability (Haziqah Che Marzuki et al., 2019; Najafi-taher et al., 2018). NEs can be prepared via an easy method and have suitable potential applications in the food industry to deliver nutraceuticals and antimicrobial agents (Azrini Nadiha Azmi et al., 2019; Food Bioprocess Technol, 2012). NE drug systems are essential for improving the delivery and bioavailability of hydrophobic drugs over free drugs (Kumar et al., 2019). NEs have a better penetration effect of the ingredients due to the small droplet size and high surface area and low surface tension of the entire emulsion system (Bouchemal et al., 2004). The surfactant concentrations are 3–10% in NEs and 20% or higher in microemulsions (Tan et al., 2016; Dehghani et al., 2017). As a result, NEs can be good candidates for medical applications. Recently, a phospholipid complex-based NE system has been reported for oral insulin delivery (Hu et al., 2019).

So far, some nanomaterials with antidiabetic properties have been reported, including silver nanoparticles (Saratale et al., 2018), zinc oxide nanoparticles (Rehana et al., 2017), gold nanoparticles (Si et al., 2017), NE of fenugreek oil (Hassan & Mujtaba, 2017), and NE of carvacrol (Hussein et al., 2017). Today, the use of plants plays a vital role in nanotechnology (Hajiashrafi & Motakef, 2018). Many medicinal plants are recommended for medical applications (Kooti et al., 2015), such as psychiatric disorders (Saki et al., 2014) and analgesics (Bahmani et al., 2014). The herbs expand the diabetes treatment in various areas such as Iranian (Hasani-Ranjbar & Mohammad Abdollahi, 2008), Indian (Saxena & Vikram, 2004), and Chinese (Wang & Wylie-Rosett, 2008) plants due to their ingredients. Fenugreek is one of the medicinal plants with many therapeutic applications (Basch et al., 2003), such as the effect on glycemia (Neelakantan et al., 2014), hypocholesterolemia (Al-Habori & Raman,

1998), blood lipid, blood glucose, and platelets (Bordia et al., 1997). Also, the antidiabetic property of fenugreek has been reported in both animal models and humans (Kumar et al., 2005). Various animal models have been studied for the antidiabetic effect of fenugreek, such as rats (Kumar et al., 2005; Mowla et al., 2009) and rabbits (Abdelatif et al., 2012). The fenugreek gum's macro- and nano/submicron emulsions of fenugreek gum have been tested in terms of stability and physical properties (Kaltsa et al., 2016). Nettle is a plant with extraordinary properties (Kregie et al., 2018), such as antidiabetic property (Ahangarpour et al., 2012), control of blood pressure and glycemic (Ghalavand et al., 2017), alpha-amylase inhibition (Rahimzadeh et al., 2014), antibacterial activity (Singh et al., 2012), hyperplasia effect (Hirono et al., 1994), and anti-inflammatory properties (Obertreis et al., 1990). Cumin (*Cuminum cyminum* L.) is an herb with biomedical activities (Srinivasan, 2018) for different applications such as antidiabetic effect (Samani Keihan et al., 2016; Abdou Mohamed et al., 2018), antioxidant activity (Abdou Mohamed et al., 2018; Bettaieb et al., 2011), glycemic control, insulin resistance and serum inflammatory factors (Jafari et al., 2017), and aldose reductase and alpha-glucosidase inhibitor (Lee, 2005a).

Nettle extract causes severe and dose-dependent inhibition of tumor necrosis factor secretion and interleukin-6 (Teucher et al., 1996). Also, caffeic-malic, which is the main phenolic component of nettle, inhibits the synthesis of cyclooxygenases in a dose-dependent manner, thus inhibiting the production of cytokines, which can lower blood sugar (Obertreis et al., 1990). Glycosylated hemoglobin is an indicator of serum protein glycosylation status in patients with diabetes. Due to its structural similarity to glucose, vitamin C can reduce the rate of hemoglobin glycosylation in patients with type 2 diabetes by competing with glucose (Gomez-Perez et al., 2010). Vitamin C is abundant in the leaves of the nettle plant. The presence of large amounts of vitamin C in nettle leaves can be one of the causes of decreased hemoglobin glycosylation in the intervention group. However, heat reduces the amount of vitamin C. The amount of vitamin C remaining in the extract and other compounds in nettle and their synergistic effects could effectively reduce the percentage of HbA1C (Tarighat Esfanjani et al., 2012). In addition, nettle contains large amounts of flavonoids (Mehri et al., 2011). Among them, quercetin from the family of polyphenols is more than other flavonoids in nettle leaves. With its antioxidant properties, Quercetin alters cellular function, and flavonoids can improve blood sugar indices (Song et al., 2005). Hydroalcoholic extract of nettle, through its antioxidant properties and inhibition of free radicals, regenerates pancreatic beta cells and thus reduces blood sugar levels in the rat (Golalipour

et al., 2006). Polyphenols typically significantly reduced fasting glucose and glycosylated hemoglobin after 45 days in diabetic rats, while not affecting glucose indices in healthy rats (Golalipour & Khorrami, 2007a). Tannins and carotenoids are also considered constituents of nettle (Kamalakkannan & Prince, 2006). Cumin fruit contains large amounts of antioxidants. Ascorbic acid, niacin, copper, and magnesium in cumin seeds have anti-diabetic effects (Talpur et al., 2005). It also delays and reduces the process of hyperlipidemia (Lee, 2005b). Numerous pharmacological studies and clinical trials have been performed on the hypoglycemic and cholesterol-lowering effects of fenugreek seeds and their various extracts. In most of these studies, the above effects have been confirmed. In addition to the physical effect of fibrous compounds, mucilage, and saponins present in the grain, which cause delay or incomplete absorption and lack of absorption of carbohydrates and cholesterol, some other compounds in the plant have been known as the leading causes of these effects. The amino acid 4-hydroxy isoleucine, the primary amino acid in fenugreek seeds, is one of the main blood sugar-lowering compounds in the plant that stimulates insulin release from the pancreas. Trigonelline, nicotinic acid, and coumarin compounds have also been reported in some studies to have an anti-diabetic effect on the plant. The critical point about the antidiabetic effects of this plant is that after consuming fenugreek seeds, the clinical symptoms of diabetes, especially polyuria, polydipsia, weakness and lethargy, and weight loss, are significantly improved. Fenugreek also decreased low-density and very-low-density lipoprotein and increased high-density lipoprotein in people with type 2 diabetes and alloxan-induced diabetic rats. The compound of saponin in fenugreek has been introduced as an anti-inflammatory, antimicrobial, and antiviral agent of the plant (Leung & Foster, 1996; Wichtl & Bisset, 1994; Barnes et al., 1996; Duke, 2002; Al-Habori & Raman, 1985). In this study, we prepared new nano-emulsions for diabetes treatment due to the studied and proven properties of these plants in improving diabetes and the synergy of these properties, which has not been done already. This article objective was to prepare O/W NE containing nettle and fenugreek extract and cumin essential oil (CEO) for antidiabetic properties and investigating cytotoxicity on Human fetal kidney cell (HEK293) cell line. This study provides important new information about NEs based on essential oils and aqueous extract for biomedical applications.

Materials and methods

Materials

All chemicals were of analytical grade. Ultrapure water was used for preparing all reagents solutions. Tween 80

and span 80 were purchased from Merck Millipore (Germany) as surfactants.

Fenugreek and nettle aqueous extracts were prepared from Adonis Gol Daro (Iran). Fenugreek and nettle in Iran were collected from Kashan in July and from Mazandaran in May, respectively. The samples were washed thoroughly with double distilled water to remove the dust particles. They were then confirmed by herbarium PMP-1741 for fenugreek (*Trigonella foenum-graecum*) and PMP-1379 for nettle (*Urtica dioica*) Tehran University. Finally, the samples were cut into pieces and dried in the presence of sunlight. For preparing the extract, 500 g of the samples was immersed in the 4 L of DD water and boiled to prepare 1 L of extract. CEO was purchased from Barij Essence (Iran) with FCL63-03 code and I951584 sample serial number.

HEK293 was prepared from the Iranian Institute of Pasteur (Iran) to investigate cytotoxicity assay. Mouse pancreatic beta-cell (RIN-5) and rat L6 myoblast cell line (L6) were obtained from the Iranian Biological Resource Center (Iran) and the Iranian Institute of Pasteur (Iran) for diabetic tests, respectively. The culture media (RPMI 1640 and DMEM/F12) were purchased from INOCLON company (Iran).

Methods

Preparation of nano-emulsion

In the preparation of mint nano-emulsion, tween 80 and span 80 have been used as surfactants (Feizi Langaroudi & Motakef, 2019). Also, in another study, span 80 was used to prepare saffron nano-emulsion (Karimi & Motakef, 2021). These materials were selected as this study's surfactants, due to the low toxicity and pharmacy uses of these surfactants (Mc Clements, 2013). To prepare nettle nano-emulsion, tween 80 and span 80 were used separately, and it was observed that span 80 did not have good immediate stability, but tween 80 was apparently stable. The same thing was repeated for fenugreek nano-emulsion, and it turned out that tween 80 did not have good temporary stability, but span 80 had good stability. Therefore, tween 80 was selected for nettle nano-emulsion and span 80 for fenugreek nano-emulsion of preparation.

According to the percentages mentioned in the research for mint nano-emulsion (Feizi Langaroudi & Motakef, 2019), nano-emulsions were prepared with 3% of different materials, and then the DLS evaluation and the sample appearance showed a sample with a medium percentage containing 95% of the extract, which is desirable and was used as a formulation for the preparation of nano-emulsions. According to the previous report for the preparation of mint nano-emulsion, the percentage of NE components is presented in Table 1 (Feizi Langaroudi & Motakef, 2019). Based on the previous article

Table 1 The percentage of NE components

Sample	Essential oil (w/w%)	Aqueous extract (w/w%)	Surfactant (w/w%)
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)

and another study on saffron nano-emulsion that used a homogenizer at room temperature (Karimi & Motakef, 2021), when we used a homogenizer to prepare a mint nano-emulsion, we first used a homogenizer. However, we used an ultrasonic bath and an ultrasonic probe due to the lack of easy access to the homogenizer and compared the results. The results show that the particle size in the method of using a homogenizer for the preparation of nettle nano-emulsion is 22 nm; in the method of using an ultrasonic bath, it is 23 nm (Fig. 1). In the end, there was no significant difference between the homogenizer and ultrasonic bath results, and we were able to use the ultrasonic bath (Parsonic7500s, Iran) as an alternative method.

Also, because in the previous article, a homogenizer was used for 30 min, and an ultrasonic bath had a maximum of 18 min at first use, it remained for 18 min, and a good result was obtained, so the method based on the ultrasonic bath was selected for 18 min. Fenugreek nano-emulsion (FN) was prepared from a mixture of CEO, surfactant, and fenugreek aqueous extract (FAE) by an ultrasonic bath for 18 min in the presence of tween 80 and span 80 surfactants. Nettle nano-emulsion (NN) was prepared exactly similar to FN, but in the presence of nettle aqueous extract (NAE). The CEO and surfactant were mixed on a stirrer, and then the aqueous extract was added slowly.

The transport and consumption of soluble medicines by the patient are a little complicated. On the other hand, the advantages of freeze-drying include no oxidation of the material, no new composition, reduced adhesion of materials, uniformity of particle size, and increased shelf life, especially in the case of drugs (Wang et al., 2017). Consequently, we used the freeze-drying method to dry the samples to make the samples into solid powder.

To perform the freeze-drying process, 100 ccs of each sample was prepared separately, poured into 10 cm plates, and placed in the freezer for 1 day. Then, we took the samples out of the freezer, covered them with foil, and made a few holes on the foil to let the solvent out. The samples were placed in a freeze dryer for 24 h. After 24 h, as the samples still contained solvent, they were

placed in the freeze dryer for another 24 h. After 24 h, the sample was placed in the machine for a total of 48 h, and the fenugreek sample was wholly dried, and the nettle sample had a little water. To dry, we covered it with a few holes covered with foil and put it in the cupboard to dry.

Evaluating of nano-emulsion

Dynamic light scattering was reported in size and size distribution (ZEN314, England). To obtain DLS, first, pour the sample into the tube for the device, and then place it in it. After adjusting the solvent (FAE and NAE for each sample) and soluble (CEO) parameters, wait for 300 s and obtain the corresponding diagram. This method was repeated three times. There is no need to dilute the sample. To study the particle size distribution after freeze-drying, first, the size of a spatula tip of each sample was poured into 5 cc of distilled water and placed in an ultrasonic bath for 10 min, and then DLS was taken from the solution. Scanning electron microscope was employed to observe the morphology and size (KYKY EM3200, China). Transmission electron microscopy was also used to observe the morphology and size of samples (Zeiss-EM10C-100 KV, Germany). The rheological properties were studied by examining viscosity based on spindle speed for samples using Rheologica Instruments AB (Lund, Sweden). The NEs were allowed to equilibrate for 72 h before evaluating their rheological properties. Rheology of flow science and deformation of currents describes the interaction between forces, deformation, and time. The setting of the rheometer is based on the formation of the structure in the time period and at the appropriate temperature based on previous study (Feizi Langaroudi & Motakef, 2019). The NEs were placed under minimum stress of 0.05 Pa and maximum stress of 550 Pa and 600 Pa. Rheograms were obtained automatically in triplicate while gradually increasing shear stress to the specified maximum stress.

In vitro studies

In this study, two types of cells were used: RIN-5 and the L6. L6 cells were purchased from the Pasteur Institute of Iran, and RIN-5 cells were purchased from the Iranian Biological Resource Center. The specific culture medium for the L6 cell line was DMEM + 2 mM glutamine 10% fetal bovine serum (FBS), and the specific culture medium for the RIN-5 cell line was RPMI 1640 + 10% FBS + 2 mM L-glutamine. Each cell was placed in its culture medium, where 1% antibiotic (100 IU/ml penicillin and 100 µg/ml streptomycin) was added to its culture medium. Then, it was maintained in a humidified incubator with 5% CO₂ at 37 °C. The cells were cultured in a flask with the required density and incubated for a while before the test.

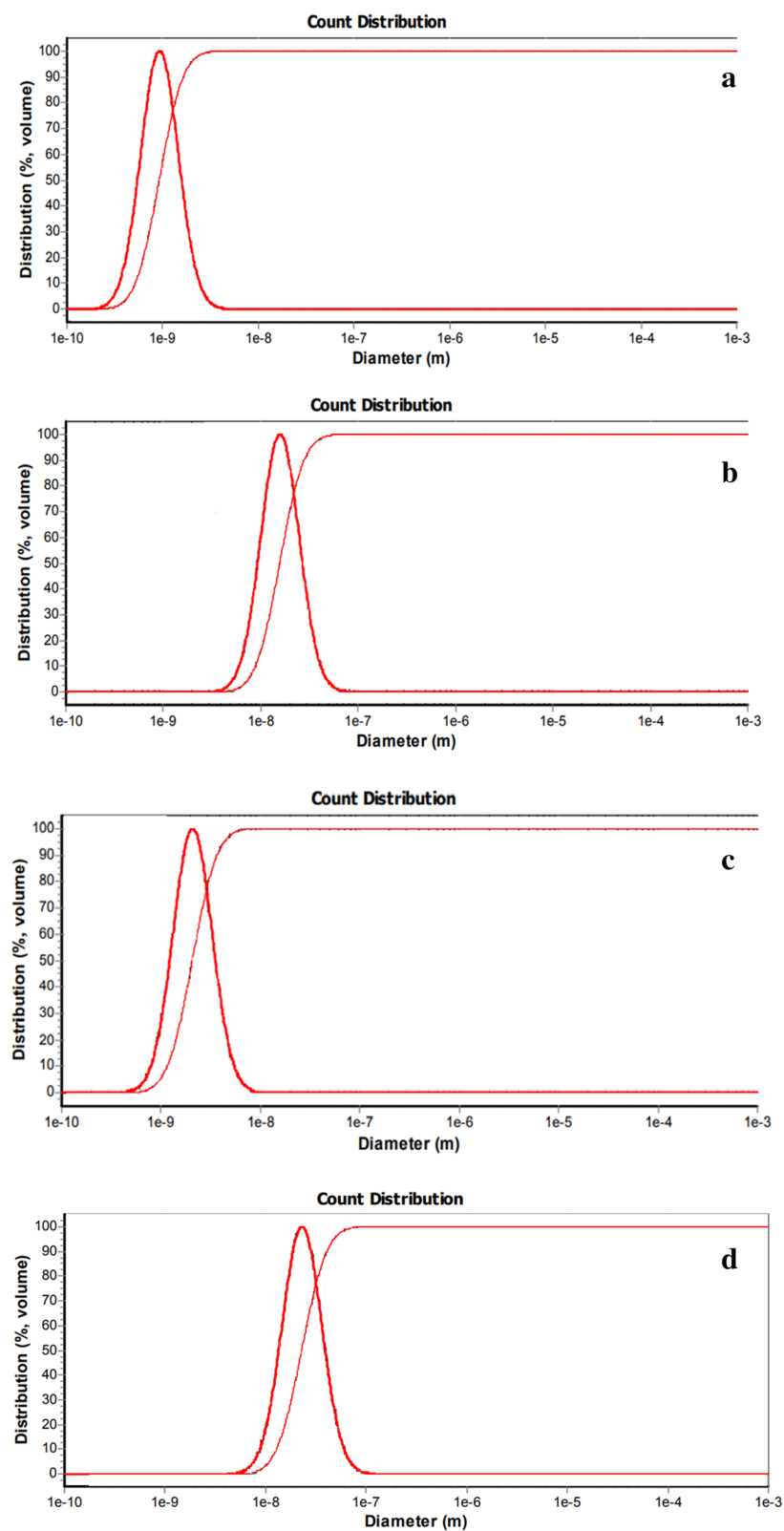


Fig. 1 DLS diagram of NN with different preparation methods. **a** 30 min homogenizer. **b** 15 min homogenizer. **c** 15 min ultrasonic probe. **d** 18 min ultrasonic bath

The cytotoxicity was investigated by HEK293 for MTT assay at three different concentrations for 1, 3, and 5 days. The cell line was purchased from the Pasteur Institute of Iran. The specific culture medium for this cell line is RPMI 1640+10% FBS. HEK293 cells were used to carry out a cytotoxic test, where we cultured 5000 cells in each of the 96-well plates with 1 ml of a specific culture medium. Then, the cells were incubated for 24 h at 37°C and 5% CO₂. After incubation, the media was changed with a new medium that contains a substance that we want to use in different concentrations (0, 0.5, 0.25, 0.12 mg/ml) for toxicity. Next, it was incubated for 24, 72, and 120 h. After this period, the supernatant medium was discarded, and adherent cells were washed by phosphate buffer solution (PBS). We added about 20 µL of MTT solution (5 mg/mL MTT bromide in PBS) and incubated it for 4 h at 37°C. The medium was removed, and the MTT formazan crystals formed by living cells were dissolved in 100 µL of dimethyl sulfoxide. The absorbance was measured at 595 nm, and the number of living cells was calculated with the standard curve. This test was then performed for RIN-5 and L6 cells at an optimal concentration obtained from the previous tests, for different samples, for 24 h.

The antidiabetic property was evaluated by two methods, including the amount of glucose absorbed by the L6 cells and the amount of insulin released from the RIN-5 cells. For evaluating the glucose absorbed amount, the L6 cells were transferred in 24-well plates at 5×10^4 cells in each well with 1 ml of their medium culture. Then, we added Krebs–Henseleit buffer (pH 7.4, 0.141 g/L of MgSO₄, 0.16 g/L of KH₂PO₄, 0.35 g/L of KCl, 6.9 g/L of NaCl, 0.373 g/L of CaCl₂·2H₂O, and 2.1 g/L of NaHCO₃) containing 0.1 % bovine serum albumin, 2 mM sodium pyruvate, and 10 mM Hepes (KHH buffer). Thereafter, two modes with low glucose and high glucose cultures were used for testing glucose intake in each environment: (1) without adding any of the samples, (2) with NE, (3) with aqueous extracts, and (4) with essential oil and incubated for 4 h. Then, we measured the concentration of glucose in the environment, reduced the initial concentration of glucose in the environment, and obtained the amount of glucose absorbed by the cell.

For evaluating the amount of released insulin, the RIN-5 pancreatic cells were transferred to 24-well plates at 2×10^5 cells in each well with 1 ml of the medium culture, with the cells cultured at 37°C and 5% CO₂ incubator. After 24 h, the cell culture medium was replaced, and the cells were placed adjacent to a new culture medium containing glucose concentration (12.5 mM glucose). Next, the cells were incubated for 3 h. After this time, the adjacent by the appropriate concentrations of cell toxicity test results from each NE, extracts, and essential oils

with 1% FBS. Then, they were incubated for 6 h. After this period, with a specific ELISA kit (Insulin ELISA kit, Ab100578, Abcam, Cambridge, UK), cell insulin levels were measured, and the level of insulin secreted for each sample was compared with the level of control insulin secretion (without any sample).

Results and discussion

Dynamic light scattering

DLS evaluated particle size and size distribution of the NE (Fig. 1). The optimum sample had a single-peak with a narrow distribution on nanometer-scale including CEO (4% w/w), FAE (95% w/w), and span 80 (1% w/w) for FN and CEO (4% w/w), NAE (95% w/w), and tween 80 (1% w/w) for NN. The mean particle diameter of optimum NE was observed at 23 nm for NN and 86 nm for FN (Fig. 1a, b). The results of the optimal percentage were similar to the previous study for different herbal nano-emulsion (Feizi Langaroudi & Motakef, 2019).

Dynamic light scattering was used to evaluate NE stability after various times 30 and 90 days included, at different temperature conditions, including 24, 4, – 4, and – 20°C. The stability of the samples was confirmed by changing the time and temperature using a DLS diagram. The mean particle diameter was 37, 50, 80, and 102 nm respectively (Fig. 1c–f) for NN and 97, 98, 105, and 127 nm (Fig. 1g–j) for FN at 24, 4, – 4, and – 20°C respectively after 30 days. Based on the results, the samples' size did not change considerably; in other words, they were stable at different temperatures. The DLS results of the optimum sample and the 30-day stability at different temperatures are summarized in Table 2. The slight reduction of particle size may be due to the intense shaking of the solution and the decrease of hydrodynamic radius before DLS analysis, while the slight increase of particle size was because of the growing phenomenon. The optimum temperature was selected based on particle size at 24°C for subsequent experiments. These results have been reported for the first time.

The stability was studied for nettle and fenugreek NEs by DLS analysis at 24°C after 90 days. DLS results showed a single-peak and a narrow distribution diagram with a size of 135 nm for NN and 115 nm for FN, which confirmed a partial change of size (Fig. 2k, l). The results were comparable to the natural anti-proliferative agent loaded self-micro emulsifying nanoparticles for potential therapy in oral squamous carcinoma (PatilAmol et al., 2019).

The effect of the freeze-drying process was investigated on the size of nettle and fenugreek NEs via DLS analysis for 2 days. DLS results showed a single-peak and a narrow distribution diagram with about 115 nm for NN and 105 nm for FN (Fig. 2m, n). Accordingly, a minor

Table 2 DLS results of the optimum sample and the 30-day stability at different temperatures

Sample	Sampling time and temperature	Number of peaks	Particle size (nm)
FN	Primary sample	1	86.3
FN	After a month at a temperature of 22–24 °C	1	98.7
FN	After a month at a temperature of 3–4 °C	1	97.9
FN	After a month at a temperature of – 3 to – 4 °C	1	105
FN	After a month at a temperature of – 20 °C	1	127
NN	Primary sample	1	23
NN	After a month at a temperature of 22–24 °C	1	37.3
NN	After a month at a temperature of 3–4 °C	1	43.1
NN	After a month at a temperature of – 3 to – 4 °C	1	79.9
NN	After a month at a temperature of – 20 °C	1	102

reduction in size results from a decline in the hydrodynamic radius.

Zeta potential was used to determine the surface charge of NE. Based on the results, the zeta potential was – 3.85 mv for NN and + 7.89 mv for FN (Fig. 3).

The more stable sample had a higher value (positive or negative) of zeta potential. The zeta potential result was according to a previous study with different nano-emulsion (Sharifi et al., 2021). The results showed low stability of the samples, but this could be due to the sedimentation of the samples, which is reversible and returns to its previous state by simply stirring or shaking the container. As shown in Fig. 4 for NN and Fig. 5 for FN, the sample was divided into two phases before shaking and returned to the previous state after shaking. Evaluations such as DLS and anti-diabetic properties were performed after shaking the sample.

Scanning electron microscopy

The SEM images showed NEs with spherical morphology (Fig. 6). According to the results, the particle size was estimated for NN about 90 nm and FN about 75 nm and confirmed DLS results (Fig. 6a, b). The result was according to a previous study with Mint nano-emulsion (Feizi Langaroudi & Motakef, 2019).

Scanning electron microscopy was employed to evaluate NE stability after various times, including 30 and 90 days, at different temperature conditions, including 24, 4, – 4, and – 20 °C. SEM results were investigated for NEs at 24 °C after 30 days (Fig. 6c, d), which confirmed the preservation of particles' size and morphology. The particle size growth was observed for nettle and fenugreek NEs after 90 days at 24 °C (Fig. 6e, f). Unlike DLS results, SEM results showed instability of the sample after 90 days, which could be due to a change in the SEM device or the operator of the device and its

lack of knowledge in drying the sample. The SEM results revealed spherical nanoparticle morphology for nettle and fenugreek NEs after the freeze-drying process for two days (Fig. 6g, h), which confirmed the size results of DLS. SEM images confirmed the stability of the samples with time and temperature change. The results have been studied for the first time.

Transition electron microscopy

The TEM results are shown for fenugreek and nettle NEs (Fig. 7) and conformed to the SEM and DLS results. The samples were observed with spherical morphologies with 34.5 nm particle size for NN and 58.3 nm particle size for FN, which confirm nano-size range of the emulsions. The morphology result was according to the TEM of a previous study for different nano-emulsion (Kumari et al., 2018).

Rheological properties

Rheology is the study of the flow of matter that deals with the deformation and flow of materials (Mehrnia et al., 2017). Rheological analysis plays a very important role in the design of release systems (Jaworska et al., 2015). Rheology tests were performed to determine viscosity of NEs at different shear rates (until 1000 s^{–1}). According to the result, the viscosity of FN was higher than that of NN at the same shear rate (200 s^{–1}); for example, the viscosity was 20 and 19 (Pas.s) at the same shear rate of 200 s^{–1} for fenugreek and nettle NEs respectively, which can be due to smaller size and a more significant number of particles in a constant volume. Knowledge of nano-emulsion rheology is essential and can indicate the shelf life of these products. The interactions between molecules, such as van der Waals, electrostatic or hydrophobic forces, have a large effect on the rheological properties. If the emulsion is a system with a high degree of dispersion, the

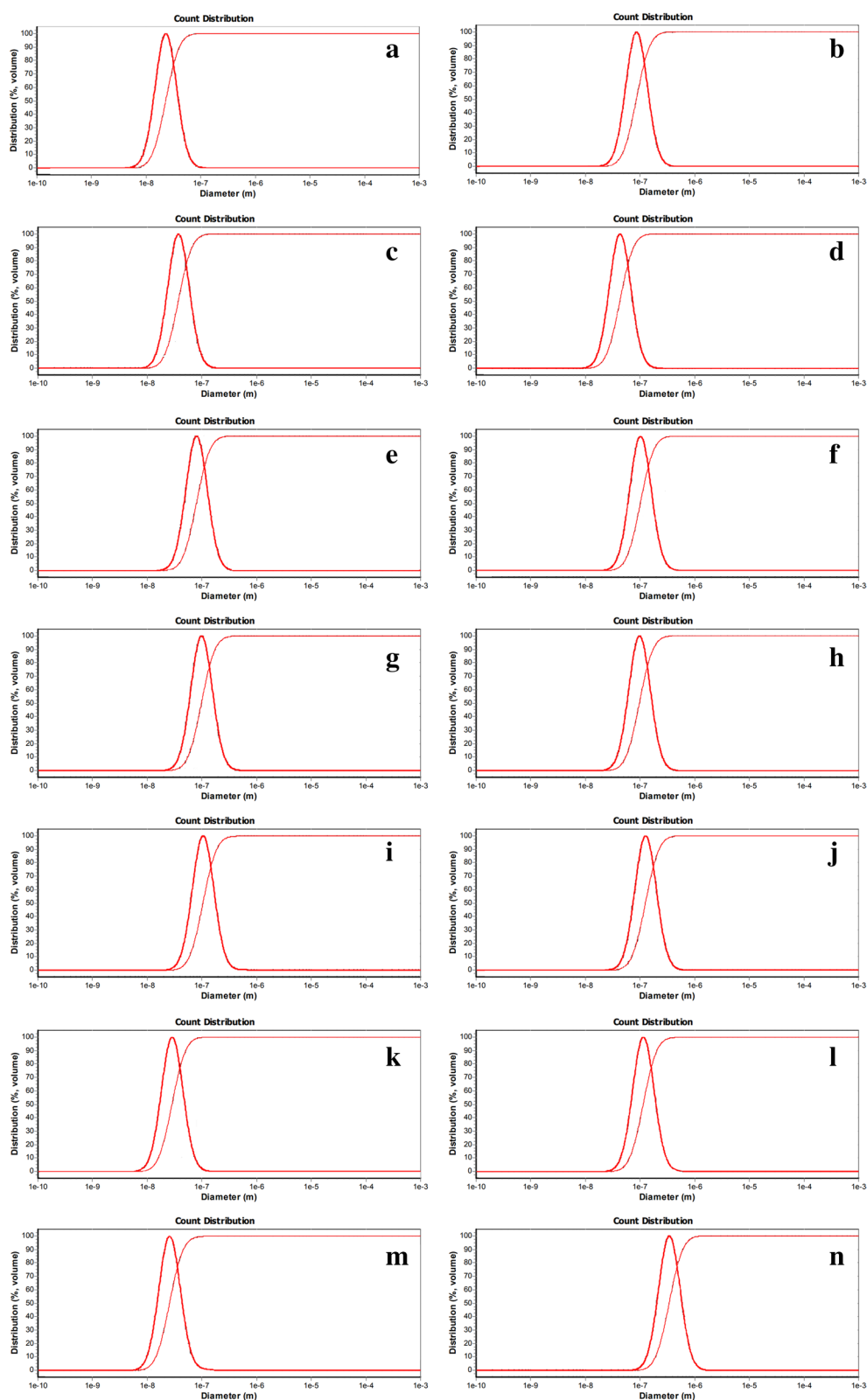


Fig. 2 DLS diagram of **a** NN in the optimum percentage, **b** FN in the optimum percentage, **c** NN after 30 days at 24 °C, **d** NN after 30 days at 4 °C, **e** NN after 30 days at − 4 °C, **f** NN after 30 days at − 20 °C, **g** FN after 30 days at 24 °C, **h** FN after 30 days at 4 °C, **i** FN after 30 days at − 4 °C, **j** FN after 30 days at − 20 °C, **k** NN after 90 days at 24 °C, **l** NE after 90 days at 24 °C, **m** NN after freeze-drying, and **n** FN after freeze-drying

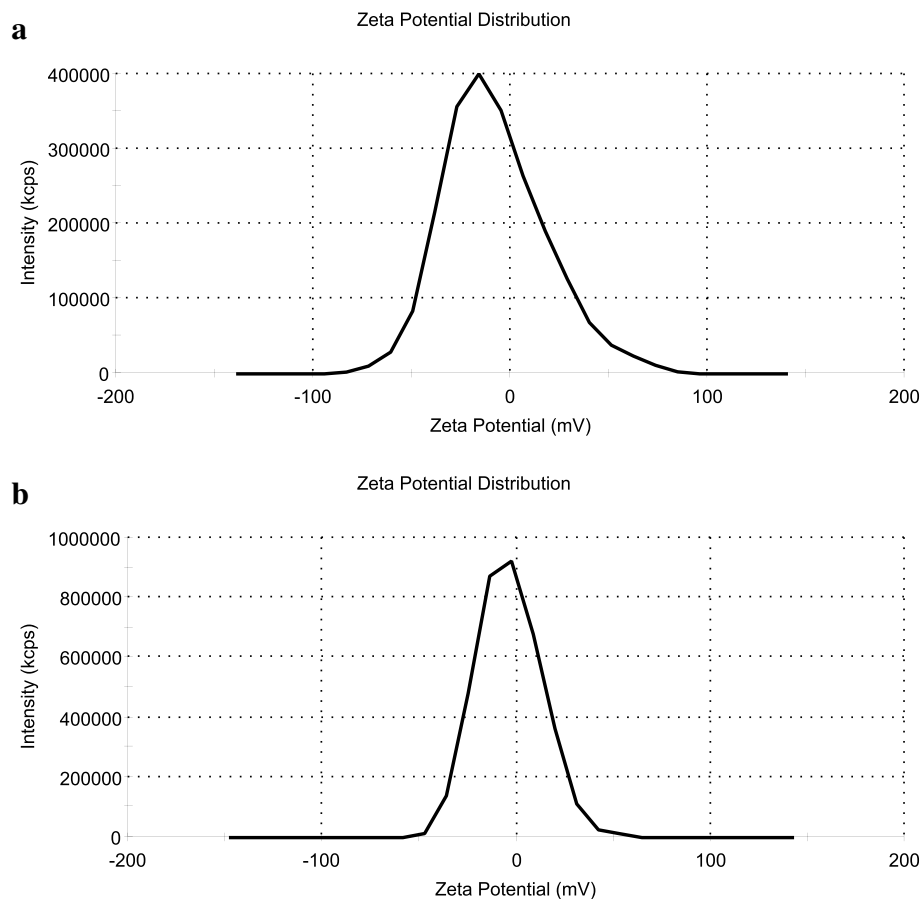


Fig. 3 Zeta potential diagram of **a** nettle and **b** fenugreek NEs

interaction between the particles is weak. However, if the number of droplets increases, the interaction between them becomes stronger and at the same time the viscosity of the whole system increases (Jaworska et al., 2015). According to the diagrams (Fig. 8), the viscosity of fenugreek nano-emulsion was higher at the same cutting speed, which indicates less stability of this. In total, both samples showed relatively high viscosity, indicating their low stability. The result concurred with a previous study (Feizi Langaroudi & Motakef, 2019). The rheological properties of NEs can be adjusted by controlling the fraction of the dispersed phase volume and the type of components (Madar & Shomer, 1990).

MTT assay

The cytotoxicity of samples was investigated by MTT assay at different concentrations (0, 0.12, 0.25, and 0.5 mg/ml) by HEK239 human cell line for three periods of time (1, 3, and 5 days) as well as RIN-5 and L6 cells for 1 day. Figure 9 displays cytotoxicity results of the NN, FN, NAE, FAE, and CEO with different concentrations and control concentration (zero) by HEK239 human

cell line. After one day, the concentrations of 0.25 and 0.12 mg/ml showed the appropriate viability of cell and the lowest cell death, but the concentration of 0.5 mg/ml was observed to increase cell death rate. After 3 days, the results revealed high cell death for the concentration of 0.5 mg/ml and low cell death for two concentrations of 0.25 and 0.12 mg/ml. There was no significant difference between the two concentrations of 0.12 and 0.25 mg/ml. After 5 days, the results indicated high cell death for a concentration of 0.5 mg/ml and low cell death and approximately equal for two concentrations of 0.25 and 0.12 mg/ml. Based on the result, concentration and time were two influential factors on cytotoxicity, and the increase of concentration and time caused the higher cytotoxicity. The result was in line with a previous study (Saratale et al., 2018). Cytotoxicity results indicated that two concentrations of 0.25 and 0.12 mg/ml were appropriate. Due to the low difference in the results of these two concentrations, the highest possible concentration for subsequent tests was selected 0.25 mg/ml.

The cytotoxicity of samples for a concentration of 0.25 mg/ml was investigated by RIN-5 and L6 cells for

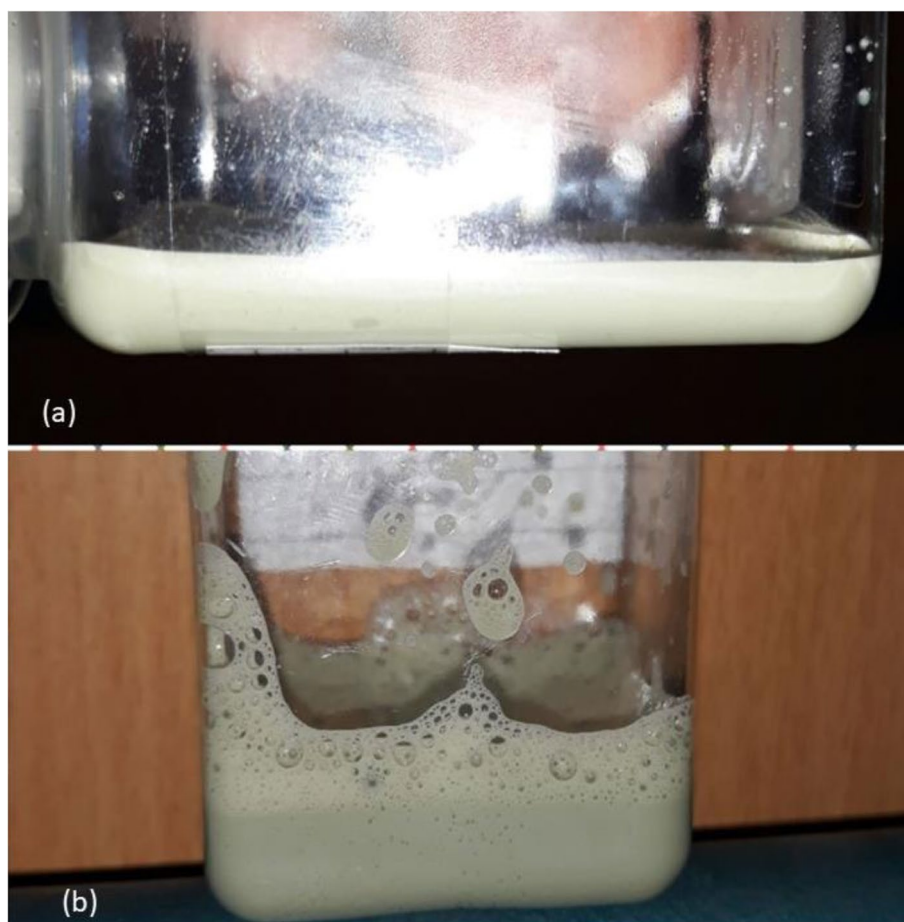


Fig. 4 NN **a** before and **b** after shaking



Fig. 5 FN **a** before and **b** after shaking

one day (Fig. 10). The results were suitable and caused a slight cell death suggesting that we could use this amount for further tests.

Antidiabetic tests

The antidiabetic tests were evaluated by two methods to measure the amount of absorbed glucose and the amount

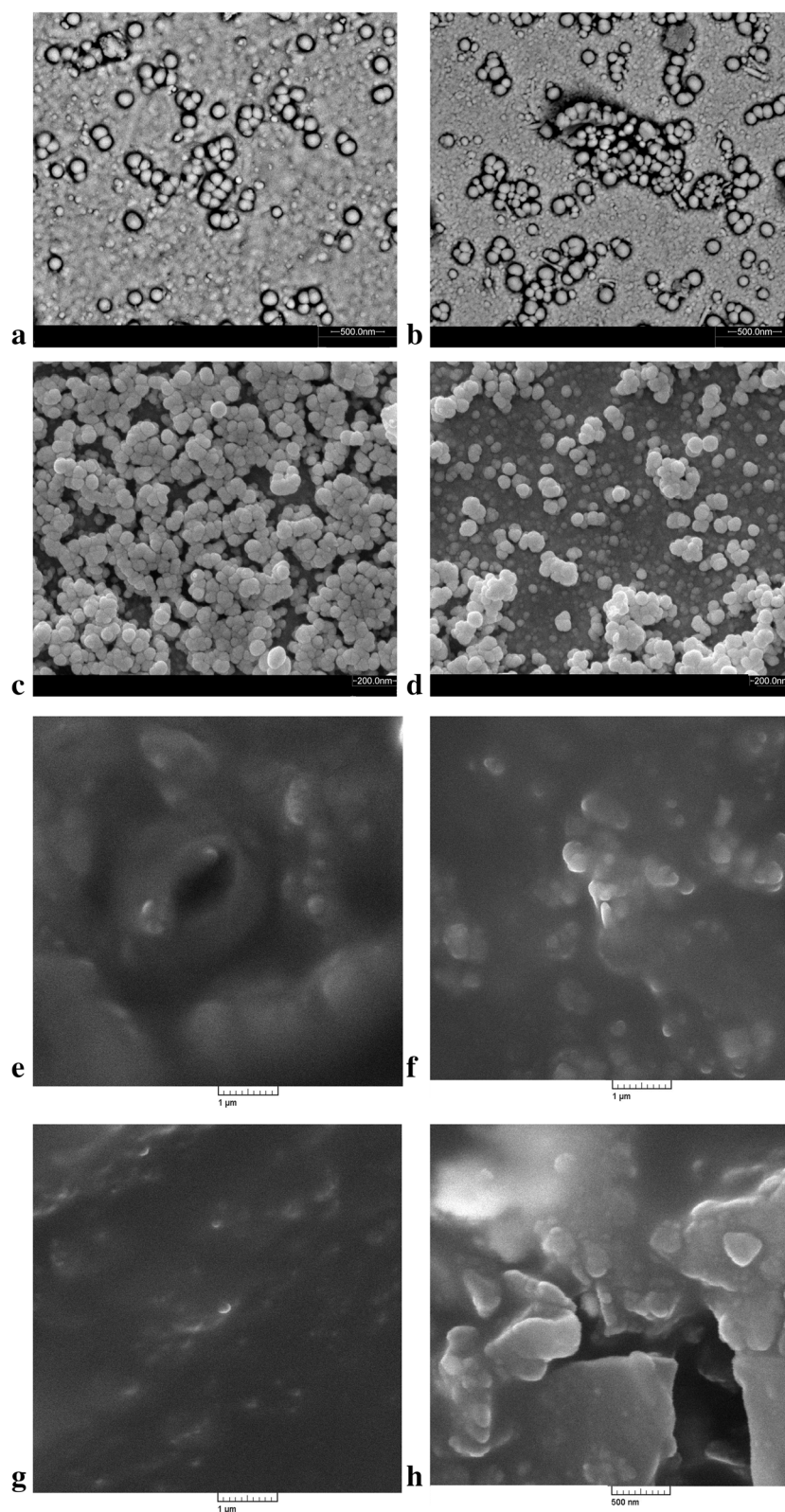


Fig. 6 SEM images of **a** NN in the optimum percentage, **b** FN in the optimum percentage, **c** NN after 30 days at 24 °C, **d** FN after 30 days at 24 °C, **e** NN after 90 days at 24 °C, **f** FN after 90 days at 24 °C, **g** NN after freeze-drying, and **h** FN after freeze-drying

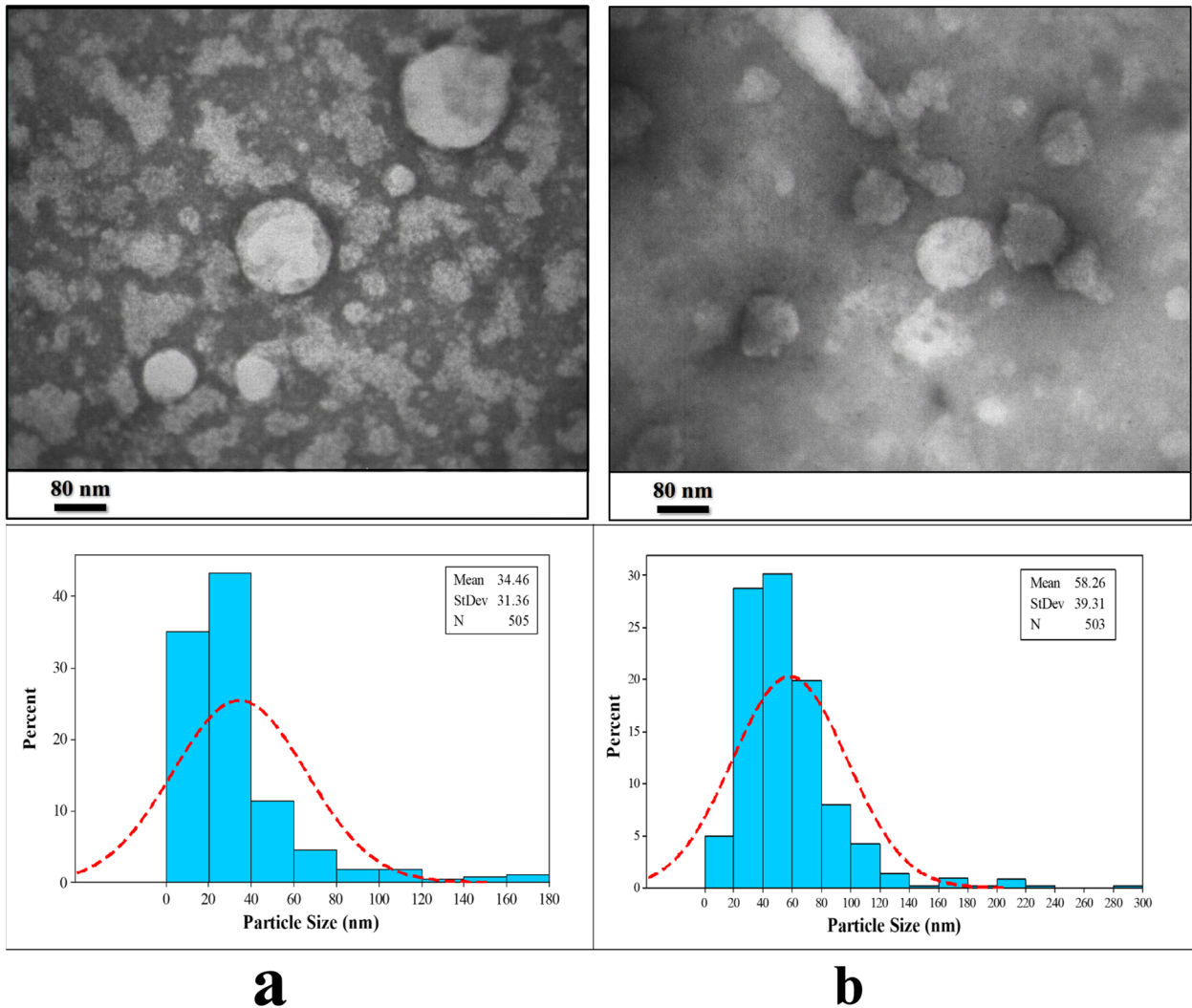


Fig. 7 TEM images of **a** nettle and **b** fenugreek NEs

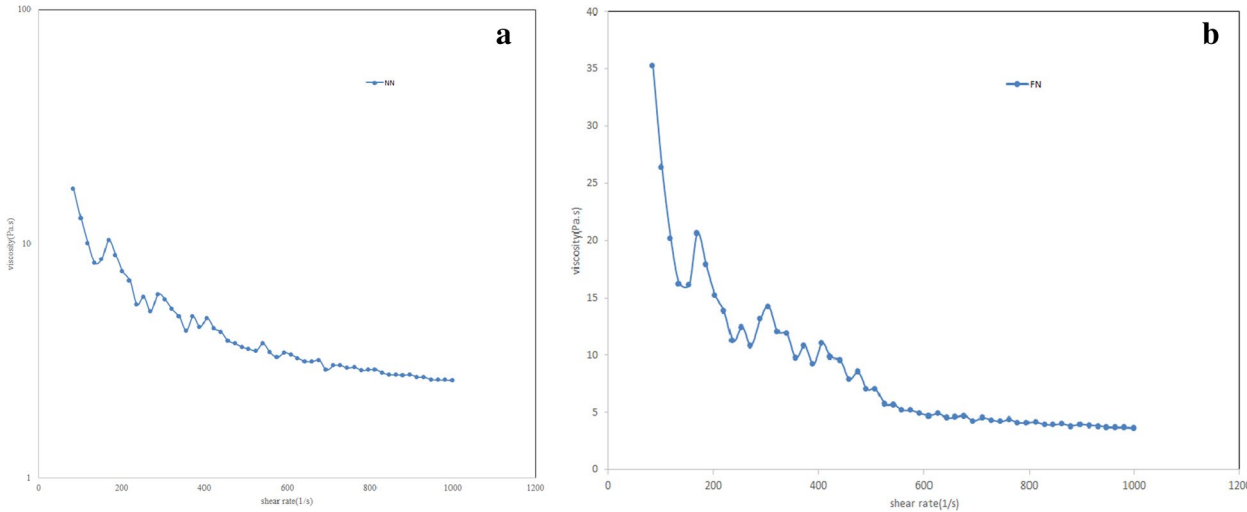


Fig. 8 Viscosity in terms of shear rate of **a** nettle and **b** fenugreek NEs

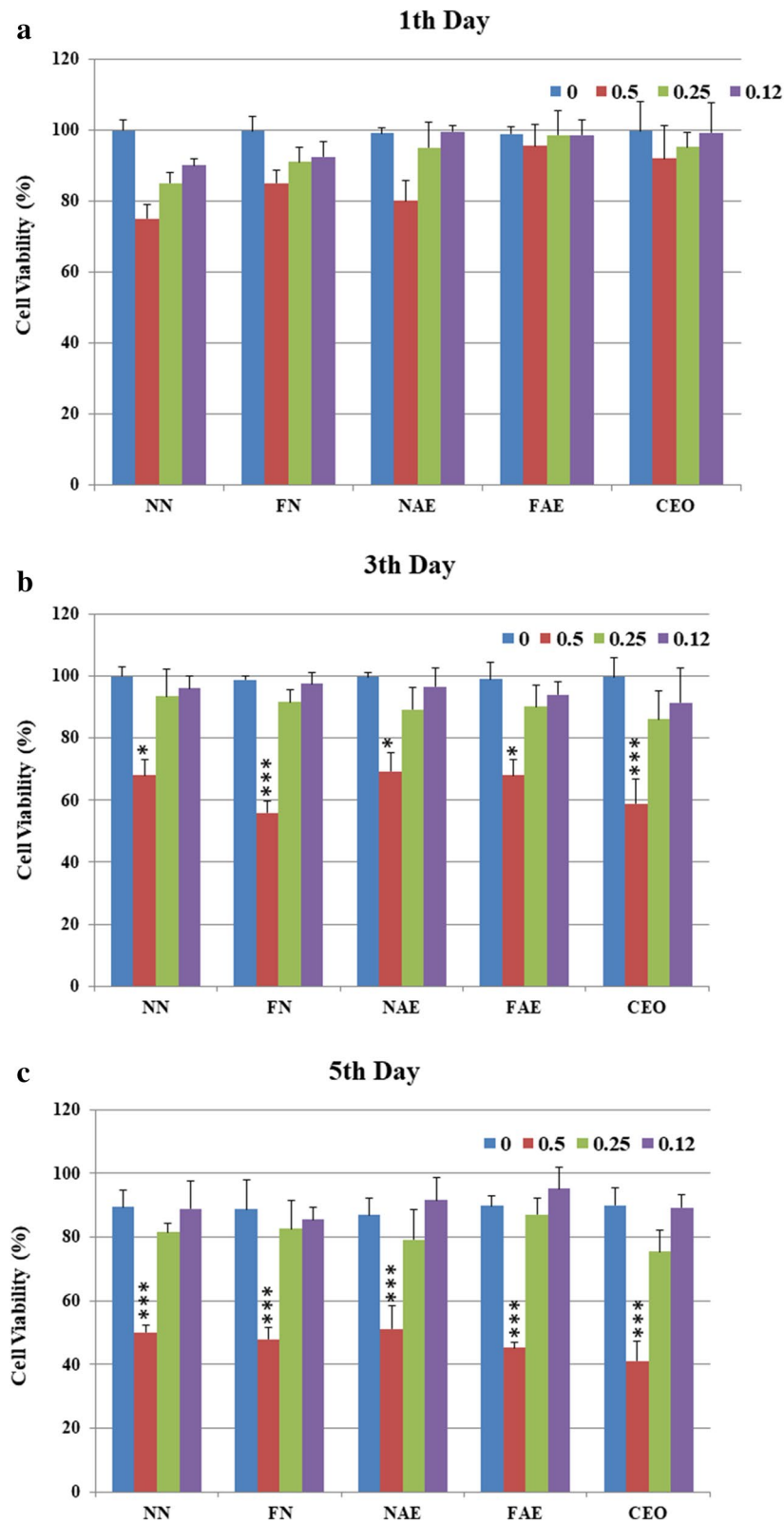


Fig. 9 The cytotoxicity curves of samples in different concentrations for **a** 1 day, **b** 3 days, and **c** 5 days by HEK239 cells

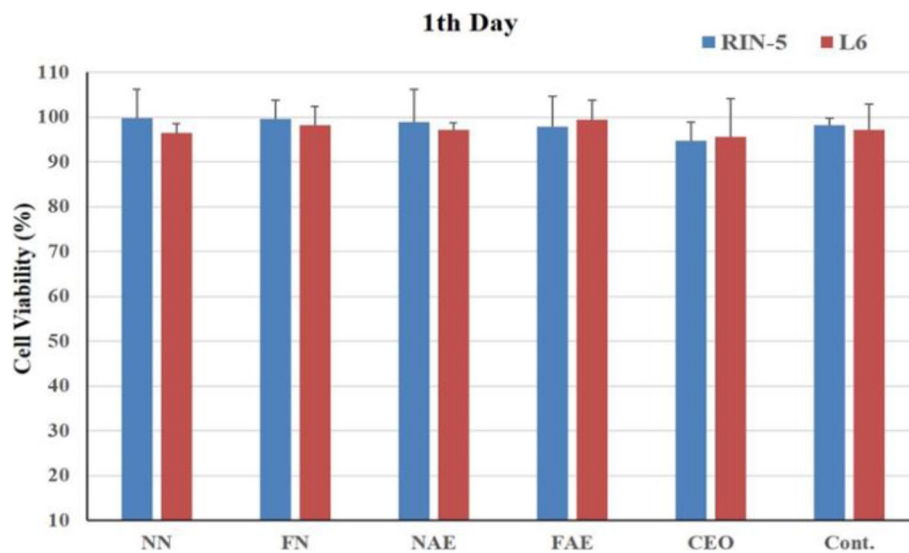


Fig. 10 The cytotoxicity curves of samples in 0.25 mg/ml for one day by RIN-5 and L6 cells

of released insulin at a concentration of 0.25 mg/ml of samples. The amount of absorbed glucose was investigated based on average glucose and high glucose by L6 cells with the difference of the entire glucose and the residual glucose in the medium after adding each sample. According to the results of both types of medium, increased glucose absorption was observed after adding FN with raising the glucose levels. Subsequently, CEO showed the highest glucose absorption by the cell. Also, the results were confirmed by the double effect of CEO with the fenugreek extract. According to the results of glucose absorption for each nano-emulsions, the extracts and essential oil were as follows:

In normal glucose:

FN (50 µg/well) > CEO (46.5 µg/well) > FAE (41.3 µg/well) > NAE (39.5 µg/well) > NN (38 µg/well)

In high glucose:

FN (96.5 µg/well) > CEO (79.2 µg/well) > NN (68 µg/well) > FAE (65.2 µg/well) > NAE (59.2 µg/well)

The glucose absorption was showed by L6 cells for FN, NN, FAE, NAE, and CEO in two media, with normal glucose and high glucose (Fig. 11).

The amount of released insulin was evaluated by RIN-5 cells for all three media, containing high glucose (12.5 mM), low glucose (6.25 mM), and no glucose. Based on the results in both high and low glucose, the level of insulin secretion significantly increased by RIN-5 cells after adding FN to the media, which indicated the excellent effect of this nano-emulsion on the control of diabetes. CEO showed the highest insulin secretion level following FN, which indicated CEO with fenugreek extract increased the control of diabetes. The effect of the

FN and CEO was enhanced with increasing glucose in the medium. According to the results, the order of insulin secretion for each nano-emulsions, the extracts, and essential oil are as follows:

High glucose:

FN > CEO > NN > FAE

Low glucose content:

FN > CEO > NN > FAE

When glucose was not present in the environment, none of the samples had any effect, and insulin levels were normal. The insulin secretion level showed in glucose-free, low glucose, and high glucose levels for FN, NN, NAE, FAE, and CEO (Fig. 12).

In this study, nano-emulsion diminished the blood sugar in vitro confirming the previous reports. The effects of FN and aqueous fenugreek extract confirmed the antidiabetic property in previous studies (Hassan & Mujtaba, 2017; Al-Habori & Raman, 1998; Bordia et al., 1997; Kumar et al., 2005; Jaworska et al., 2015; Sauvaire et al., 1998). The effect of CEO confirmed the reduction of blood sugar in previous studies (Samani Keihan et al., 2016; Abdou Mohamed et al., 2018; PatilAmol et al., 2019; Jagtap & Patil, 2010). The nettle hydroalcoholic extract had a good effect on blood sugar in previous findings (Ahangarpour et al., 2012; Golalipour & Khori, 2007b). In this research, the NN and NAE did not have a very significant effect on insulin secretion and glucose absorption, showing the influential role of hydroalcoholic extract compared with aqueous extract on antidiabetic properties. The observed effects can be due to the active ingredients in the plants, which have been increased by the preparation of nano-emulsions.

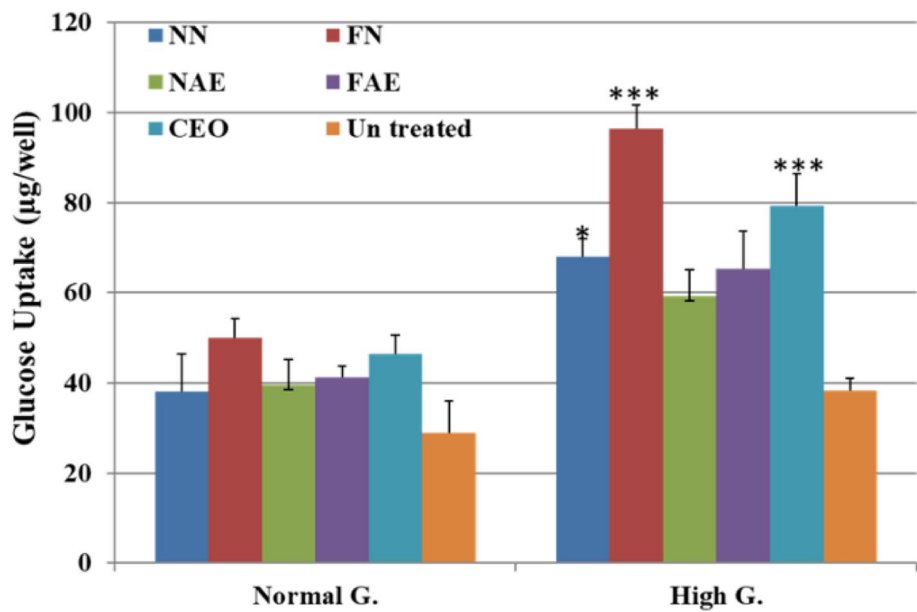


Fig. 11 The glucose absorption of samples in 0.25 mg/ml for one day by RIN-5 and L6 cells

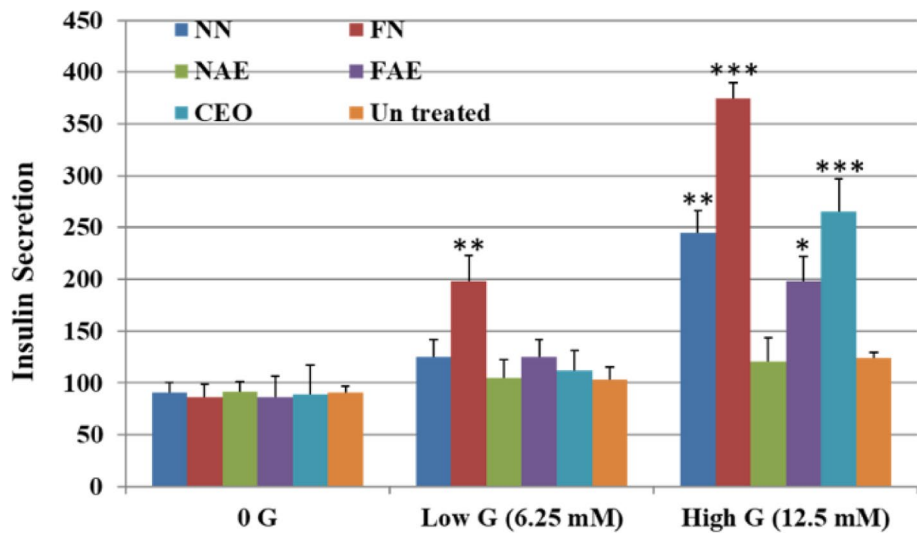


Fig. 12 The insulin secretion level by RIN-5 cell culture in glucose-free, low glucose, and high glucose levels for samples

Conclusion

This study demonstrated a nano-emulsion based on the plants with a simple and cost-effective method. The optimum sample contained NAE (95% w/w), tween 80 (1% w/w), and CEO (4% w/w) for NN and FAE (95% w/w), span 80 (1% w/w), and CEO (4% w/w) for FN with low cytotoxicity for HEK239, RIN-5, and L6 cells. DLS and SEM results revealed a single peak with a narrow distribution diagram and nanoparticles with spherical

morphology, respectively. According to TEM results, the particle size was observed 58.3nm for FN and 34.5nm for NN. Based on DLS and SEM analyses, samples had good and suitable stability after different times (30 and 90days), temperatures (24, 4, − 4, and − 20°C), and freeze-drying. Given the advantages of FN, it can be a good candidate for diabetes control and treatment as a pharmaceutical and food agent with low toxicity than chemical drugs in the future.

Abbreviations

Cumin: *Cuminum cyminum*; CEO: Cumin essential oil; DLS: Dynamic light scattering; Fenugreek: *Trigonella foenum-graecum*; FE: Fenugreek extract; FAE: Fenugreek aqueous extract; FN: Fenugreek nano-emulsions; FBS: Fetal bovine serum; HEK293: Human embryonic kidney cells; NE: Nano-emulsion; NPs: Nanoparticles; *Urtica dioica*: Nettle; NE: Nettle extract; NAE: Nettle aqueous extract; NN: Nettle nano-emulsion; O/W: Oil-in-water; O/W/O: Oil in water in oil; PBS: Phosphate buffer solution; L6: Rat L6 myoblast cell line; RIN-5: Mouse pancreatic beta-cell; SEM: Scanning electron microscopy; SLNs: Solid lipid nanoparticles; TEM: Transmission electron microscopy; W/O: Water-in-oil; W/O/W: Water in oil in water.

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Authors' contributions

Ms. Sara Javadi is a student and Dr. Motakef-Kazemi is supervisor and Raheleh Halabian is an adviser. The authors read and approved the final manuscript.

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We agree to make the data and materials available.

Declarations

Competing interests

The authors declare that they have no competing interests.

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