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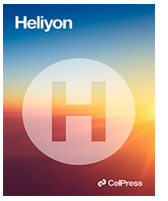

Research article

Laboratory study: Synthesis and optimization of nano nisomes containing *Bunium persicum* essential oil and investigating its toxicity on *Trichomonas vaginalis* parasite and HFF cell line

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A R T I C L E I N F O

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Bunium persicum
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A B S T R A C T

The use of nanotechnology can reduce the challenges facing the use of herbal compounds in the fight against infectious agents. The aim of the present research is to produce nano niosomes containing *Bunium persicum* essential oil with high efficiency in the temperature and acidity of the living environment of *Trichomonas vaginalis* parasite and to investigate its toxicity on this parasite. First, Essential oil compounds were identified using GC-Mass. Then six niosomal formulations were made using Tween 40, 60, and 80 and cholesterol by thin film method. Three formulations that have more suitable particle size, zeta potential, and essential oil release and encapsulation efficiency were selected by MTT method to investigate the toxicity on HFF (Human foreskin fibroblasts) cell line. The formulation with lower toxicity was optimized using DSPE-mPEG(2000) polymer. Encapsulation efficiency, particle size, zeta potential, release of essential oil (in temperature and acidity similar to *Trichomonas vaginalis* living environment), particle morphology and toxicity of optimal formulation (on HFF and *Trichomonas vaginalis*) were investigated. At the end, the stability of the optimized nanoparticles was studied for 120 days. 12 chemical compounds including γ-Terpinene, Cuminic aldehyde and Para-cymene were identified *Bunium persicum* essential oil. The optimized formulation has a particle size of 159.73 nm, a zeta potential of −25.1 mV and an encapsulation efficiency of 63.11 %, which has a slow and continuous release at the similar temperature and acidity as *Trichomonas vaginalis*. Niosomal nanoparticles have a spherical shape and a smooth surface and have little toxicity on the HFF cell line. Also, the toxicity of nano niosomes containing essential oil on *Trichomonas vaginalis* is higher than free essential oil in all concentrations. The optimized niosomal nanoparticles have good stability because their physicochemical properties have changed very little during 120 days. In conclusion optimized Niosomal formulation containing *Bunium persicum* essential oil compared to free

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essential oil can have a higher efficiency to deal with *Trichomonas* parasite in laboratory conditions.

1. Introduction

Trichomonas vaginalis is a single-celled parasite, eukaryotic and belonging to the Protista kingdom. This flagellar single cell is the most common non-viral sexually transmitted parasite that can cause Trichomoniasis infection in the urogenital tract, in such a way with approximately 248 million new cases detected annually [1]. Trichomoniasis infection is associated with clinical symptoms such as the production of foul-smelling secretions in the urogenital tract, feeling of pain when urinating, etc, which can lead to preterm birth, acquisition, premature rupture of the amniotic sac and low birth weight baby. Also this disease can increase risk of human immunodeficiency virus (HIV) [2,3]. The ideal temperature and pH for the growth of *Trichomonas vaginalis* is 35–37 $^{\circ}C$ and 4.9 to 7.5, respectively that with the change of these conditions, its growth and development will be impaired. This non-viral parasite is not naturally able to survive in vaginal acidity, but any factor that can cause the vaginal environment to become alkaline can increase the risk of trichomoniasis infection. Metronidazole is commonly used to treat *Trichomonas vaginalis* infection, which is associated with widespread side effects such as headache, vomiting, nausea, dry mouth, dizziness, and in some cases potential carcinogenicity, teratogenic effects on the fetus and drug resistance has also been reported for this drug [4–7]. Therefore, it is necessary to use natural compounds such as herbal compounds with high effectiveness and few side effects. The use of medicinal plants and the compounds extracted from them has become more widespread today. It seems that low side effects, easier access, naturalness and in some cases their lower price compared to chemical drugs are among the reasons for humans to return to the use of medicinal plants [8]. Numerous studies also show that plant compounds have antifungal [9], antibacterial [10], antioxidant [11,12] and anti-tumor [12,13] properties. *Bunium persicum* is an annual herbaceous plant with a hollow, smooth stem that belongs to a family of plants called *Apiaceae*. This plant has white hermaphrodite flowers that grows in Eastern Europe, Asia and especially Eastern Iran. In traditional medicine, *Bunium persicum* plant and its extracted compounds are used for the purpose expectorants, appetizers, anti-muscle cramps, stomach strengtheners, etc [14,15]. Phytochemical studies also show that in the essential oil extracted from *Bunium persicum*, there are active compounds such as gamma terpinene, cuminaldehyde, limonene, para-cymene, beta-pinene and caryophyllene, which these compounds cause antifungal, antiparasitic, antibacterial and anti-cancer properties in *Bunium persicum* essential oil [16–20]. For example, Mehni et al., in 2015 reported that *Bunium persicum* can have antifungal effects against Candida albicans vaginalis [21]. Also, Siyadatpanah et al., in 2023 showed that liposomal essential oil of *Bunium persicum* has good anti-proliferative effects on *Trichomonas* parasite [1]. However, on one hand Due to the significant prevalence of trichomoniasis in different communities and the need for new treatments with minimal side effects and maximum effectiveness, on the other hand, the presence of antimicrobial active compounds in *Bunium persicum* essential oil, it seems that *Bunium persicum* essential oil can be effective in the appropriate treatment of this disease. But the use of plant compounds associated with important challenges such as high oxidation, low absorption, low effectiveness, which has limited their use in various applications [22,23]. Therefore, it is necessary to provide new models to deliver essential oils and other plant compounds to the target cells. It seems that nanotechnology can solve some of the problems of delivering herbal compounds to target cells by improving drug delivery [24]. Delivery systems based on nanomaterials improve the efficiency of drug delivery and increase the effectiveness of treatment. Nanocarriers can be divided into inorganic and organic groups, that organic nanocarriers, including liposome and niosome, are more effective in drug delivery [25]. Nano niosomes are single or multi-layered structures composed of a non-ionic surfactant, which is an amphiphilic molecule, and cholesterol, which acts as a stabilizer. The structure of these nanocarriers is very similar to liposomes and they can carry hydrophilic and hydrophobic drugs [26–28]. Compared to liposomes, niosomes have higher stability, less toxicity, cheaper manufacturing method, higher biodegradability, biocompatibility, and higher encapsulation efficiency, which has made niosomes a popular drug carrier [29–32]. Also, the high stability of niosomes vesicles without the need for any external modification and low production cost are other advantages of niosomes compared to liposomes [33]. Considering the many side effects of chemical drugs, the anti-parasitic effects of *Bunium persicum* essential oil and the high potential of niosomes in drug delivery, the aim of this research is to fabricate and physicochemically characterize and evaluate the stability of niosomal nanocarriers containing *Bunium persicum* essential oil to combat *Trichomonas vaginalis* parasite.

2. Materials and methods

The present research is a type of laboratory study that was carried out during 6 months at Shahid Sadoughi University of Medical Sciences in Iran.

2.1. Materials

For the present study, Bunium persicum plant was collected from Bahabad mountains of Yazd province. Tween 40, 60, and 80, cholesterol, Dimethyl sulfoxide (DMSO) and MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) were supplied Sigma-Aldrich Company, DSPE-mPEG2000 (distearoyl phosphoethanolamine-polyethylene glycol) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Isopropyl, chloroform, phosphate buffered saline (PBS) were purchased from Merck, Germany.

2.2. Essential oil extraction

Bunium persicum plant was collected from *Bahabad* mountains of Yazd province during the fruit production season (seeding) and its species was approved by botanists of the Faculty of Natural Resources and Desertology of Yazd University. In the present study, for the essential oil extraction of *Bunium persicum* seed the distillation method in water by Clevenger apparatus was used. For this purpose, each time, transfer 100 g of powdered *Bunium persicum* seeds with 250 ml of distilled water to a 500 ml balloon and the balloon was connected to a Clevenger and essential oil extraction was done for 4 h. Finally, the produced essential oil was removed from the Clevenger apparatus and collected for the next steps.

2.3. Investigating the composition of Bunium persicum essential oil

Identification of essential oil compounds was done by gas chromatography method. For this purpose, the gas chromatograph column (Shimadzu, model 9A) was set at 50 °C for 5 min. Then the temperature was increased to 250 °C and both injection and detection were done at 290 °C. Helium gas with a linear velocity of 32 cm/s was the carrier of the column. The composition of the essential oil was identified using retention index and mass spectra of the compounds and their comparison with the standard mass spectra available in computer libraries and authoritative sources.

2.4. 2.4. preparation of niosomal system containing Bunium persicum essential oil

Bunium persicum -loaded nano-niosomes were developed and analyzed for vesicular size, zeta potential, EE% and contentious release parameters. To optimize these results and evaluate the optimal parameters following schemes were performed.

- Assessment of the influence of different types of surfactants (Tween 40, Tween 60 and Tween 80) and in different molar ratios of surfactants: cholesterol was executed on drug-loaded nano-niosome.
- Selecting the optimum molar ratios of Tween 40: cholesterol, Tween 60: cholesterol and Tween 80: cholesterol and their evaluation in terms of cytotoxicity MTT assay.
- After achieving the best formula from the point of particle size, zeta potential, EE%, release and cytotoxicity assay, 5 % DSPE-PEG 2000 was added to niosomes.
- The release pattern of pegylated niosomes containing essential oil was analyzed at different pHs of 5.5, 6.5 and 7.4 to simulate the pH of *Trichomonas vaginalis* environment.
- Investigating the toxicity of PEGylated nano niosomes containing essential oil on the parasite *Trichomonas vaginalis*

The Niosomal system containing *Bunium persicum* essential oil was prepared by a thin layer hydration method using surfactants and cholesterol according with concentrations similar to Table 1 along with *Bunium persicum* essential oil. For this purpose: First, surfactants, cholesterol and *Bunium persicum* essential oil were dissolved in chloroform solvent at 45 °C on a rotary (Heidolf, Germany) and a thin film was prepared under vacuum. Then hydration was performed by adding distilled water for 40 min at 55 °C. The prepared nanoparticles were then reduced in size using bath sonicate (Grant XB6, England) for 40 min. Finally, the nanoparticles were passed through 0.45 and 0.22 μm filters to homogenize [19]. The dose of essential oil was 1 mg/ml for all of the formulations and the L/D ratios were kept at 10. Then the prepared formulations were maintained at 4 °C.

2.5. Physical characterization of Bunium persicum– loaded nano-niosoms

The particle size, poly dispersity index (PDI) and zeta potential of the nanoparticles obtained from the selected formulation were measured using a zeta sizer device (model: HORIBA) at 25 °C, an angle of 90° and a wavelength of 657 nm. The morphology of nanoparticles was investigated using a field emission scanning electron microscope (FE-SEM) (Sigma VP model from ZEISS, Germany and equipped with EDS, Mapping, WDS and EBSD detectors from Oxford Instruments, England)

2.6. Determine the maximum absorption wavelength (λmax) and plot calibration curves

Spectrophotometry was used to determine the maximum absorption wavelength of *Bunium persicum* essential oil and draw a

Table 1
Chemical compounds used in the structure of Niosomal formulations.

Formulation Number	Lipid/Drug	Cholesterol %	Tween 40 %	Tween 60 %	Tween 80 %
F_1	10	25	75	–	–
F_2	10	30	70	–	–
F_3	10	25	–	75	–
F_4	10	30	–	70	–
F_5	10	25	–	–	75
F_6	10	30	–	–	70

calibration graph. In this method, a stock solution of essential oil with a concentration of 1 mg/ml was prepared in PBS and isopropyl. Then, the absorption spectrum was read by a spectrophotometer (Epoch, USA) in the range of 200–500 nm. The wavelength at which the maximum absorption occurred was considered as the maximum absorption wavelength. After that, different concentrations of essential oil were prepared in PBS and isopropyl solvents. using the absorption spectrum obtained from different concentrations at the maximum absorption wavelength, the calibration curve of essential oil was drawn in PBS and isopropyl buffer, and the normalized equation of the line in PBS and isopropyl was calculated using the calibration curve [34].

2.7. Calculating the encapsulation efficiency and the release rate of essential oil from niosomic systems

At the first to calculate the efficiency of essential oil encapsulation, unencapsulated essential oil (free essential oil) was removed. Then, the niosomal system was dissolved in isopropyl with a ratio of 20:1 to break the wall of nano-niosomes. Then, the absorption of niosomal essential oil was measured with a spectrophotometer, and the encapsulation efficiency of each formulation was calculated according to the calibration chart of the essential oil in isopropyl and using equation (1).

$$\text{Entrapment Efficiency (\%EE)} = \frac{\text{Encapsulated drug concentration (mg/ml)}}{\text{Primary used drug concentration (mg/ml)}} \times 100 \quad \text{Eq (1)}$$

The essential oil release was investigated at a temperature of 37 °C and at pH of 5.5, 6.5 and 4.7. Dialysis bag method was used to evaluate essential oil release. In this method, a certain amount of niosomes containing essential oil was poured into the dialysis bag. Then the dialysis bag was placed next to PBS for 48 h. Within 48 h and at intervals of 0.5, 1, 3, 6, 12, 24 and 48 h, a certain amount of PBS was removed around the bag and its absorption was measured by a spectrophotometer. At the end, by using the obtained absorptions and referring to the calibration chart of essential oil in PBS, the release rate of essential oil was calculated [22,35].

2.8. Cell culture and investigation of the toxicity of primary niosomal systems containing essential oils

The purpose of this stage is to investigate the toxicity of formulations containing Tween 40, 60 and 80, which have the higher encapsulation efficiency and suitable release. Since the niosomal formulations are designed for external use and if confirmed, they will be placed in the vicinity of healthy cells, at this stage the toxicity of these nanoparticles obtained from these formulations on the healthy HFF cell line was investigated by the MTT method. First, the healthy HFF cell line was supplied from Pasteur Institute (Tehran, Iran) and Cultivated in sterile flasks with DMEM medium, containing 10 % FBS along with penicillin and streptomycin antibiotics in an incubator with a temperature of 37 °*C* and 5 % carbon dioxide. In the MTT test, HFF cells were cultured with a concentration of 10^4 in each well in a 96-well plate for 48 h. HFF cells were treated with 6.25, 12.5, 25, 50, 100, 200, 400 and 800 μg/ml of nano-niosome containing essential oil for 48 h to check the toxicity of nano-niosome systems. After the desired treatments, 20 μL of MTT solution with a concentration of 5 mg/ml was added to each well and incubated for 4 h. After that, the supernatant was removed and 150 μL of DMSO was added to dissolve the formazan crystals. Absorbance at 570 nm wavelength was recorded using ELISA reader and finally, according to equation (2), the percentage of cell viability was calculated.

$$\text{Cell viability \%} = \frac{\text{Average absorption in the test group} - \text{Average absorption in culture medium}}{\text{Average absorption in the control group} - \text{Average absorption in culture medium}} \times 100 \quad \text{Eq (2)}$$

2.9. Optimization and characterization of the selected nano-niosome formulation

According to the encapsulation efficiency, the rate of essential oil release from the niosomal system, the size and zeta potential of nanoparticles and the toxicity of formulations F1 to F6 on the HFF cell line, one of the systems were selected for optimization and further experiments. Then selected formulation was optimized with 5 % DSPE-mPEG (2000) (PEG) and the encapsulation efficiency, the particle size, PDI and zeta potential of the optimum system was measured. Considering that *Trichomonas* lives in pH 4.9 to 7.5, the release of essential oil from the selected niosome formulation was investigated at 37 °C temperature and pH 5.5, 6.5 and 7.4 within 48 h and according to the calibration chart of essential oil in PBS, the graph of essential oil release was drawn within 48 h. Also, the toxicity of selected PEGylated formulation containing essential oil in concentrations of 6.25, 12.5, 25, 50, 100, 200, 400 and 800 μg/ml on HFF cell line was investigated by MTT assay.

2.10. Predictive model of drug release kinetics

The release of essential oil from the PEGylated formulation was estimated using zero-order, first-order, Higuchi and Korsmeyer-Pepps mathematical models [27]. Also, nonlinear regression analysis was fitted by MATLAB software and R^2(Regression coefficient) was calculated for each model.

2.10.1. Zero-order model

Drug dissolution from niosomes and released slowly. The equation can be represented as below:

$$C_0\text{-}C_t = K_0 t$$

Where *Ct* is the drug amount released in time t, C_0 is the initial drug amount in the solution and *K0* is the zero-order kinetic model

constant [27].

2.10.2. First-order model

The first-order kinetic has also been utilized to describe drug absorption and/or elimination. The drug release formula that followed this kinetic can be seen below:

$$\mathrm{LogC} = \mathrm{LogC_0} - \frac{Kt}{2.303}$$

where *C0* is the initial drug concentration, *Kt* is the rate constant, and t is the time [27].

2.10.3. Higuchi model

The Higuchi kinetic model is shown by the equation

$$Q = K_H \times t^{0.5}$$

Q is the drug amount released in time t, and *KH* is the kinetic model constant [27].

2.10.4. Korsmeyer-Peppas model

Korsmeyer derived a simple equation that explained drug release from a polymeric system from the below equation:

$$\frac{M_t}{M_\infty} = Kt^n$$

M_t = The amount of essential oil release at time t
M_∞ = The amount of essential oil release in infinite time
K= Fixed release Korsmeyer-Peppas
n = Korsmeyer-Peppas release exponent
t = Release Time [27].

2.11. Functional group analysis

Fourier transform infrared (FTIR) spectroscopy technique was used to investigate the interaction between the synthesized niosome and *Bunium persicum* essential oil. In this method, FTIR spectra of free form of *Bunium persicum* essential oil, niosome containing *Bunium persicum* essential oil and blank synthesized niosome were obtained separately. For this purpose, first 1 mg of each sample in a ratio of 1–100 added to potassium bromide (KBr) and then, the samples were located in a hydraulic press to form the pellets. Each sample was analyzed by FT-IR spectrum instrument (Brucker, Germany) at a wavelength of 400–4000 cm^{-1} and its functional groups were identified (25)

2.12. Investigating of free essential oil and PEGylated nano niosomes containing essential oil on the parasite Trichomonas vaginalis

Trichomonas vaginalis parasite was obtained from the laboratory of parasitology department of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The parasite was extracted from the patients of Isfahan health centers, which was approved by the ethics committee of Isfahan University of Medical Sciences, Isfahan, Iran for obtaining biological samples. (Code of ethics: refer to number 1396.851). For this purpose, the sample extracted from each individual was transferred to TYI-S-33 culture medium and incubated at 37 °C for 24 h. After the incubation time, in order to check the presence of parasites, one drop of each culture medium was examined with a light microscope at 40 magnifications. After culturing a large amount of *Trichomonas vaginalis* parasite, 100 μL of cell suspension was added to each well of a 96-well plate. Then the cells were incubated for 24 h at 37 °C until the number of parasites reached about 106 parasites per microliter. Parasites in the wells were treated with 2, 4, 8, 16, 32, 64, 125 and 250 μg/ml of Niosome system containing essential oil and free essential oil (not Niosome) for 24 h. After the treatment time, 20 μl of MTT solution with a concentration of 5 μg/ml was added to each well and incubated for 4 h. In order to dissolve the formazon crystal, 150 μL of DMSO were added. Absorbance at 570 nm wavelength was recorded using a ELISA reader (Epoch model, BioTek, USA) and at the end according to equation (2), the percentage of cell viability was calculated and the IC50 of free essential oil and the system containing essential oil was measured.

2.13. Stability study of selected PEGylated system containing essential oil

During 120 days, the optimized nano-niosomes containing essential oil were kept at a temperature below 4 °C. In order to investigate the physicochemical stability of nanoparticles containing essential oil, the encapsulation efficiency of nanoparticles, the release of essential oil from nanoparticles, the size and zeta potential of nanoparticles, the morphology and toxicity of nanoparticles were investigated during 120 days. For this purpose, every 30 days the particle size, zeta potential and encapsulation efficiency was analyzed. At the end of 120 days, the morphology of nanoparticles, the release of essential oil from nanoparticles at 37 °C temperature and pH 5.5, 6.5 and 7.4 and the toxicity of nanoparticles containing essential oil was investigated.

2.14. Statistical software

In this research, Excel 2016 software was used to calculate the mean and standard deviation, and Graphpad Prism version 9 software was used to perform two-way ANOVA test, and MATLAB version 2015 software was used for mathematical modeling calculations.

3. Results

3.1. Chemical composition of Bunium persicum essential oil

The chemical compounds in *Bunium persicum* essential oil were identified using GC-Mass. The most important compounds identified in *Bunium persicum* essential oil are Camphene, 1-limonene, γ-Terpinene, Para-cymene, Trans-Decalone, acetylphenylcarbinol, Cuminic aldehyde, Silicic acid. γ-Terpinene with 21.86 %, Cuminic aldehyde with 17.28 % and Para-cymene with 6.21 % are the most abundant compounds in *Bunium persicum* essential oil.

3.2. Selection the most suitable formulation for optimization

3.2.1. Determination of Lambda max and standard curve of Bunium persicum essential oil in isopropyl and PBS

Absorption spectrum of *Bunium persicum* essential oil at wavelengths of 200–500 nm showed that the essential oil has the highest absorption at the wavelength of 245 nm (Fig. 1 A). For calculate the encapsulation efficiency, the calibration chart *Bunium persicum* essential oil was drawn in isopropyl (Fig. 1 B). According to this diagram, the line equation of this diagram is Y = 9.5047X-0.2962, which has a regression coefficient of $R^2 = 0.9994$. Based on this graph, the encapsulation efficiency of each formulation is calculated and reported in Table 2. Also, the calibration chart *Bunium persicum* essential oil in PBS was drawn in order to calculate the amount of essential oil release, and its line equation and regression coefficient are Y = 3.9976X - 0.0916 and $R^2 = 0.9997$, respectively (Fig. 1 C). The amount of essential oil released during 48 h from nanoparticles obtained from F1 to F6 formulations is reported in Table 2.

3.2.2. Effect of surfactant type and molar ratio of surfactant: cholesterol on essential oil nano-niosome formulations

The encapsulation efficiency, release rate, the size and zeta potential of nanoparticles obtained from each formulation are reported

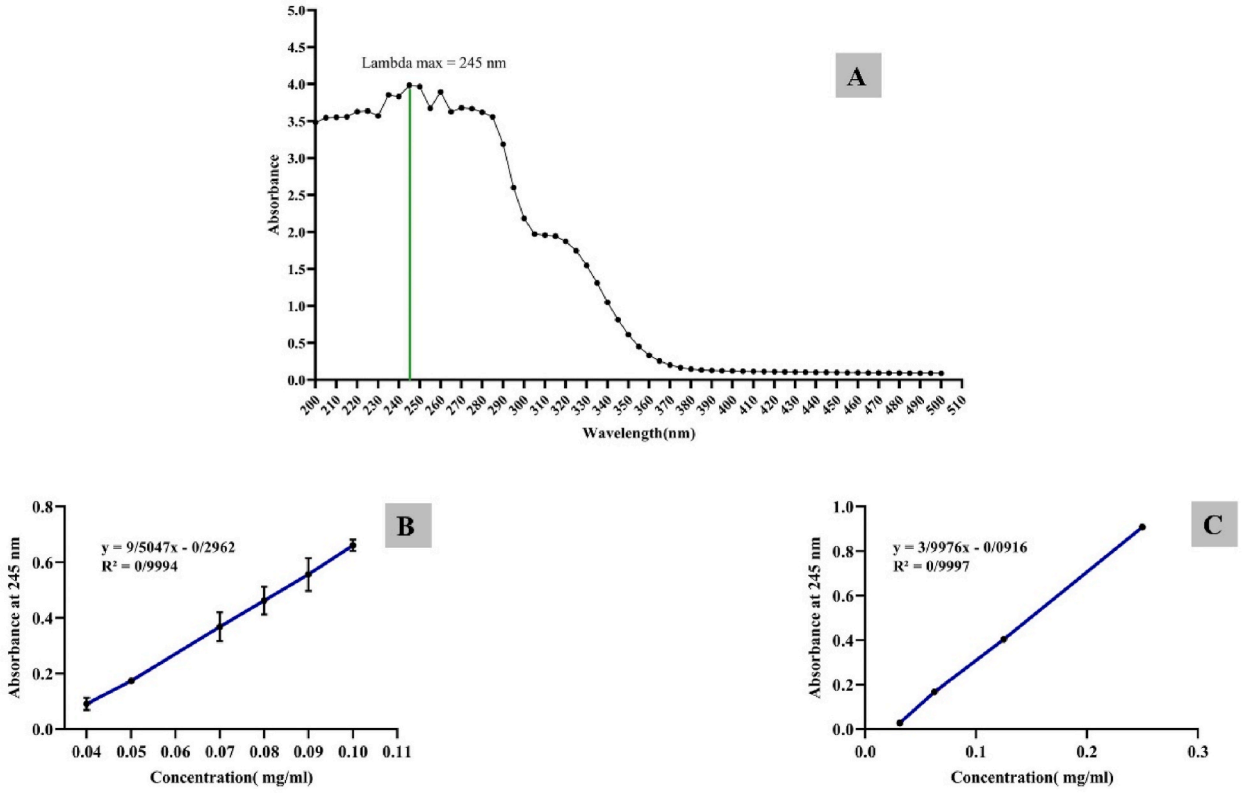

Fig. 1. Absorption spectrum of *Bunium persicum* essential oil at wavelengts of 200–500 nm (A) Calibration diagram *Bunium persicum* essential oil in isopropyl (B) and Calibration diagram *Bunium persicum* essential oil in PBS (C).

Table 2
Encapsulation efficiency (EE%), size, zeta potential and essential oil release rate from nanoparticles obtained from formulations F1 to F6.

Formulation	EE%	Size(nm)	PDI	Zeta Potential (mV)	Release %			
					6 h	12 h	24 h	48 h
F_1	51.68 ± 3.22	141.11	0.132	−27.4	43.6 ± 2.07	52.75 ± 2.43	60.13 ± 2.77	62.81 ± 2.91
F_2	49.71 ± 6.12	140.58	0.130	−25.5	40.02 ± 3.75	48.62 ± 14.76	57.1 ± 2.65	60.35 ± 1.65
F_3	55.13 ± 1.78	149.27	0.140	−24.8	52.94 ± 3.27	63.09 ± 2.89	70.93 ± 3.74	76.23 ± 8.62
F_4	56.87 ± 4.45	150.78	0.156	−26.8	53.67 ± 3.2	65.37 ± 5.88	69.4 ± 2.7	75.85 ± 3.09
F_5	56.11 ± 5.62	148.12	0.137	−27.3	57.20 ± 4.48	68.11 ± 8.21	72.41 ± 3.74	78.41 ± 5.66
F_6	53.33 ± 4.20	149.15	0.138	−26.1	55.35 ± 3.14	64.22 ± 4	70.17 ± 3.62	75.71 ± 4.01

in Table 2. As shown in Table 2, formulations F5 and F4 have the highest encapsulation efficiency with 56.87 ± 4.45 % and 56.11 ± 5.62 %, respectively, and formulation F2 has the lowest encapsulation efficiency with 49.71 ± 6.12 %. The zeta potential of nanparticles is negative in all formulations, and formulation F1 has the most negative zeta potential with −27.4 mV. However, the difference between the most negative and the most positive zeta potential is 2.6 mV. The particle size in all formulations is below 200 nm, and formulation F2 with 140.58 nm has the smallest size and formulation F4 with 150.78 nm has the largest particle size. Also, the difference in particle dispersion index between the largest size and the smallest size is 0.026. However, the poly dispersity index for all formulations is less than 0.16. The release of essential oil in all formulations is slow and continuous within 48 h. In the first 12 h after the release, all the formulations except the F2 formulation had more than 50 % essential oil release, and at the end of 48 h, the F5 formulation showed the highest release rate with 78.41 ± 5.66 %.

3.3. Cytotoxicity study

Among the formulations containing Tween 40, F1 formulation, among the formulations containing Tween 60, F3 formulation and among the formulations containing Tween 80, F5 formulation had more encapsulation efficiency and more suitable essential oil release. Therefore, the toxicity of these three formulations was measured on HFF cells. According to Fig. 2, all formulations show concentration-dependent toxicity and toxicity increases with increasing concentration. However, this concentration-dependent toxicity increase is not significant for the formulations containing Tween 60 and 80 ($P > 0.05$, but it is significant for the formulation containing Tween 40 ($P < 0.05$.). Also, the lowest level of toxicity is related to empty niosomes made with Tween 80 at a concentration 6.25 μg/ml and the highest toxicity is related to niosomes containing essential oil made with Tween 40 at a concentration of 800 μg/ml (Fig. 2 A).

3.4. Characterization of optimized formulation

According to the data reported in Table 2 and the toxicity reported in Fig. 2, formulation F5, which has higher encapsulation efficiency, proper release, and lower toxicity, was optimized with 5 % DSPE-mPEG (2000) (PEG) and selected for subsequent tests.

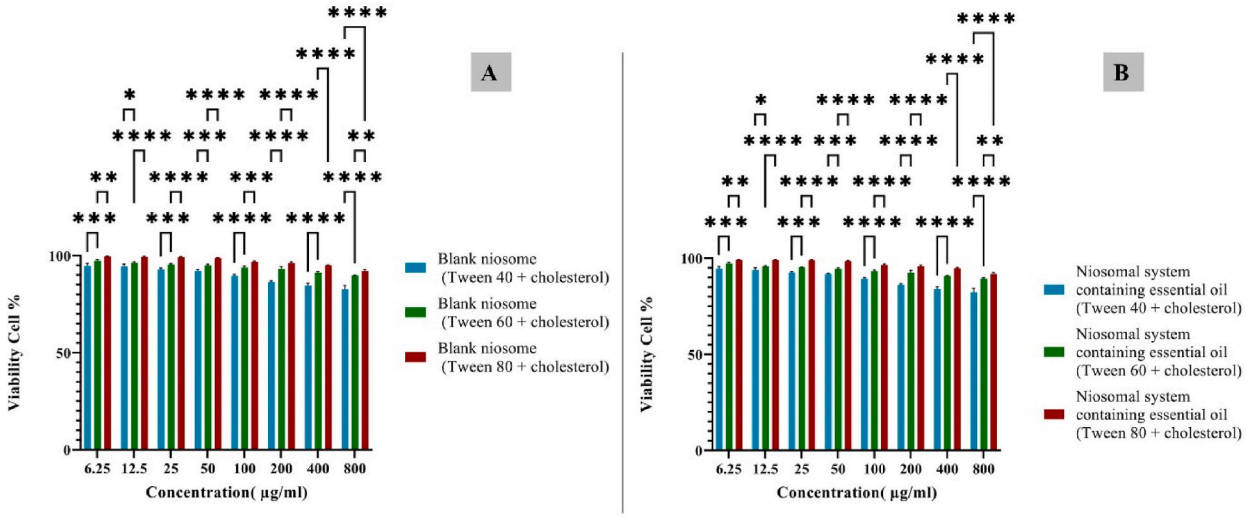

Fig. 2. Survival analysis and biocompatibility comparison of F1(tween 40: cholesterol), F3(tween 60: cholesterol) and F5(tween 80: cholesterol) formulations on HFF cell line: blank niosome (A); niosome containing Bunium persicum essential oils (B). (Significance: **, *** and ****$P < 0.05$ and ns = non-significant (

3.5. The encapsulation efficiency and the release assay of the optimized system (F5-PEG)

The encapsulation efficiency of the PEGylated formulation (F5-PEG) is 63.11 ± 2.73 %. Also, Fig. 3 A shows the release pattern of essential oil from the PEGylated system at 37 °C and pH 5.5, 6.5 and 7.4. According to this graph, the release of *Bunium persicum* essential oil from nano-niosomes decreased with increasing pH. The maximum release of essential oil in 48 h is 76.27 % at pH = 5.5, 70 % at pH = 6.5 and 66.33 % at pH = 7.4. The significance of the difference in the release of *Bunium persicum* essential oil from the optimized formulation is reported in Fig. 3 B.

Also, the results of fitting the mathematical models on the experimental data obtained from the release of the essential oil at different pH (before checking the stability) are reported in Table 3.

3.6. Physical characterization of the particles obtained from the optimal formulation (F5-PEG)

The size of nano-niosomes obtained from the optimized formula is 159.73 nm, the poly dispersity index is 0.143 (Fig. 4), and its zeta potential is −25.1 mV (Fig. 5). According to the image of the field emission scanning electron microscope (FE-SEM), the nanoniosomes obtained from the optimal nanoniosome formula (F5-PEG) are formed and no accumulation of particles is seen.(Fig. 6).

3.7. Fourier transform infrared spectroscopy (FT-IR)

Fig. 7 shows the FTIR graph of *Bunium persicum* essential oil, niosome system without essential oil (blank niosome) and niosome system with essential oil. In the FTIR graph of *Bunium persicum* essential oil, the index peaks 296.69 cm^{-1} and 2872.21 cm^{-1} are characteristic of stretch CH of alkanes group and peak 2818.06 cm^{-1} is characteristic of CHO out of plan. The index peak of 1690.26 cm^{-1} is characteristic of C=O stretch of aldehyde group and the peak of 1575.72 cm^{-1} is characteristic of C=O stretch of carboxylic acid group. Peak 1427.77 cm^{-1} is characteristic of C–C stretch in aromatic ring and peak 1382.08 cm^{-1} is characteristic of CH from alkane group. Also, the index peaks of 1305.57 cm^{-1} and 1210.61 cm^{-1} are characteristic of C–O in the ester and the peak of 772.18 cm^{-1} is characteristic of C–H out of plan in the aromatic ring. In the FTIR graph of niosomal system without essential oil, 4 peaks of 3326.18 cm^{-1}, 2130.79 cm^{-1}, 1637.54 cm^{-1} and 623.24 cm^{-1} are observed, which respectively correspond to C–H stretch of alkyne group, C–H stretch of alkyne group, C=C stretch of alkyne group and C–H of alkyne group. Also, in the FTIR graph of niosomal system containing essential oil, the index peaks of 3240.41 cm^{-1}, 1634.14 cm^{-1} and 952.08 cm^{-1} are observed, which are respectively characteristic of OH in carboxylic acid, NH amide and aliphatic NO. Comparison of the FTIR graph of the system without essential oil and the system with essential oil shows that both have similar peaks, which indicates that there was no significant interaction between the system and the essential oil. On the other hand, the peaks created in the FT-IR spectrum of the system containing essential oil compared to the system without essential oil, have undergone slight changes in location, which is a proof of the confinement of the essential oil inside the niosome.

3.8. Toxicity of nanoparticles containing Bunium persicum essential oil obtained from optimal formulation (F5-PEG)

The toxicity of optimized nano-niosomes was measured on the HFF cell line. Based on Fig. 8, nanoparticles in different concentrations have little toxicity on HFF cells. Although the toxicity of nanoparticles containing *Bunium persicum* essential oil has increased with increasing concentration, the difference in toxicity between different concentrations is not significant (P > 0.05).

The toxicity results of the niosomal system containing *Bunium persicum* essential oil and free *Bunium persicum* essential oil show that these two substances have concentration-dependent toxicity for *Trichomonas* parasites, and with the increase in the concentration of

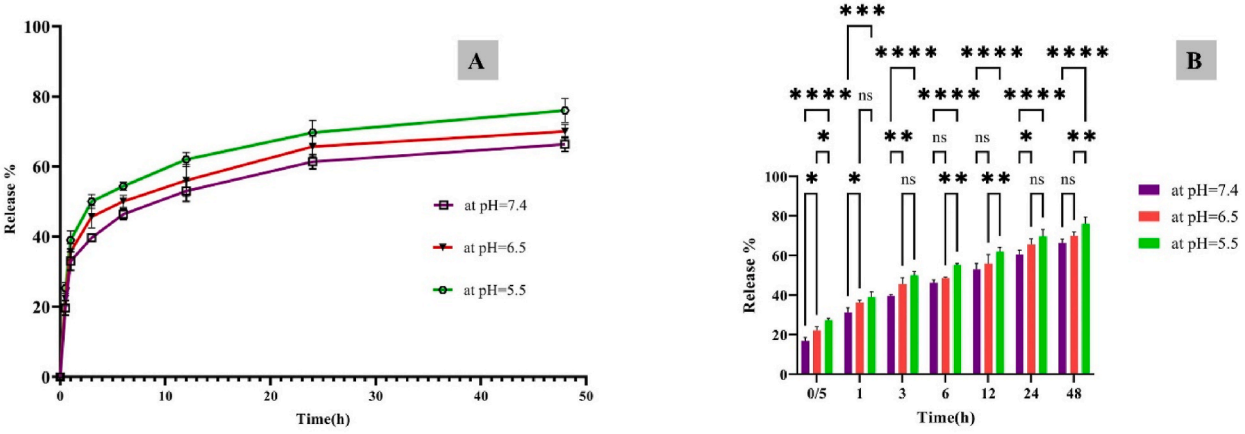

Fig. 3. The drug release profile; the release of essential oil from the optimized formulation with PEG within 48 h (A); significant comparison of the difference in essential oil release in different pH (B). (Significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns = non-significant (

Table 3
The release of kinetic models and obtained parameters for the niosomal formula (before checking the stability).

	pH-7.4	pH = 6.5	pH = 5.5
Zero order	$R^2 = 0.627$	$R^2 = 0.599$	$R^2 = 0.574$
First order	$R^2 = 0.759$	$R^2 = 0.749$	$R^2 = 0.739$
Higuchi	$R^2 = 0.855$	$R^2 = 0.833$	$R^2 = 0.0816$
Kors-peppas	$R^2 = 0.984$	$R^2 = 0.981$	$R^2 = 0.985$
	n = 0.19	n = 0.19	n = 0.19
	K = 33.8	K = 33.8	K = 37.29

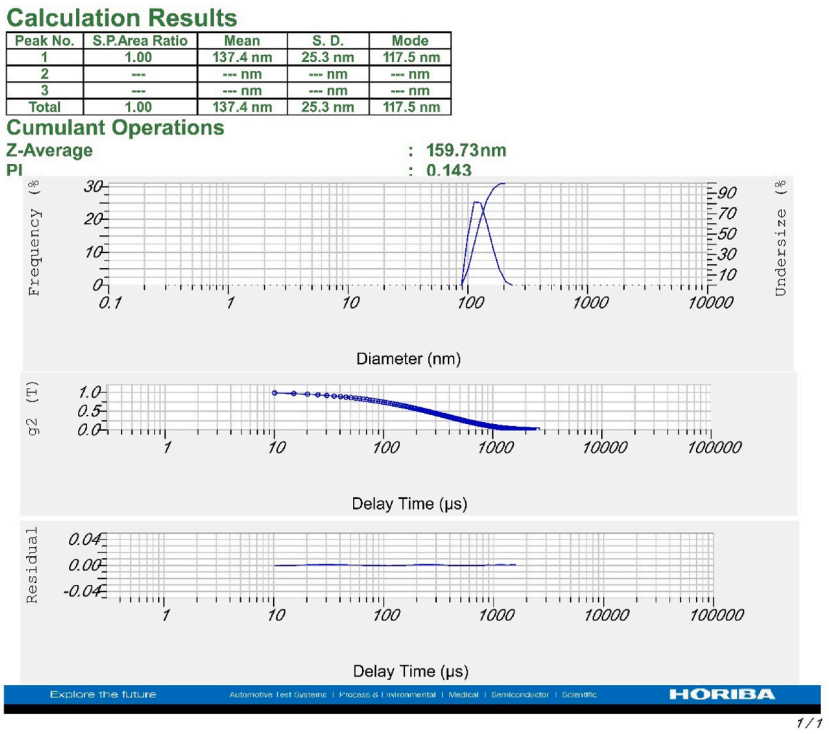

Fig. 4. The particle Size of the optimized formulation.

free essential oil and niosomes containing essential oil, the survival rate of the parasite has decreased. Also, the toxicity of nano niosomes containing essential oil is higher than free essential oil in all concentrations. This difference in toxicity between free essential oil and niosomal system containing essential oil is significant in all concentrations except for concentrations of 2 and 4 µg/ml (Fig. 9). IC50 within 24 h for nano niosomes containing essential oil and free essential oil was 11.01 and 16.09 µg/ml, respectively.

3.9. Stability examination

The stability of optimized nano-niosomes obtained from F5-PEG formulation was measured during 120 days. Zeta potential, particle size and encapsulation efficiency were measured every 30 days, which are shown in Table 4. During 120 days, the encapsulation efficiency has decreased by 21.39 % (Fig. 10 A). Also, the zeta potential of nanoparticles has become more positive by −7 mV and the size of the particles has increased by 8.37 nm, which is a small change considering the storage of nanoparticles for 4 months (stability time)((Fig. 10 B). The release of *Bunium persicum* essential oil from nano niosomas was investigated after 120 days and its graph was drawn (Fig. 10 C). According to Fig. 10 C, the pattern of essential oil release at different pHs after 120 days is similar to the release of essential oil before the stability measurement, although the amount of essential oil release has increased compared to before the stability measurement in all pHs. Also, the results of fitting the mathematical models on the experimental data obtained from the release of the essential oil at different pH (after checking the stability) are reported in Table 5. The electron microscope image shows that the shape of the nanoparticles did not change after 120 days. (Fig. 11).

After 120 days, nano niosomes were evaluated on HFF cell line and the results are reported in Fig. 12. As before the stability test, nanoniosomes at all concentrations had very little toxicity on the healthy HFF cell line. Although, as before stability measurement, the toxicity of nano niosomes depends on the concentration, but the difference in toxicity in different concentrations is not significant.

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-25.1 mV	-0.000132 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -25.1 mV
Electrophoretic Mobility Mean : -0.000132 cm²/Vs

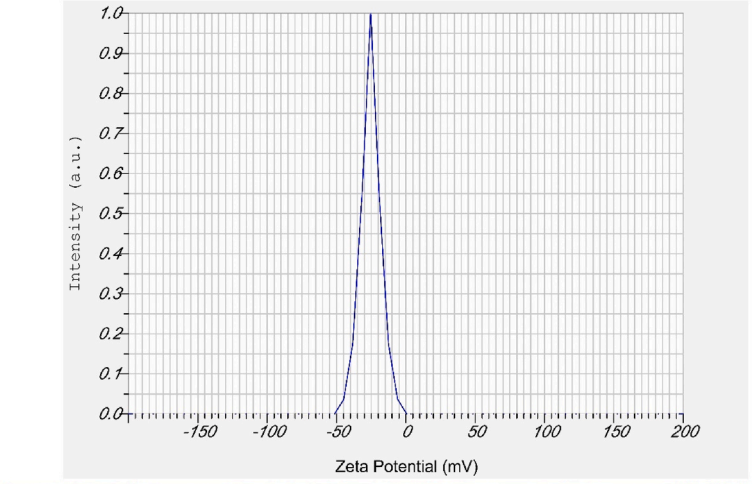

Fig. 5. The zeta potential of the optimized formulation.

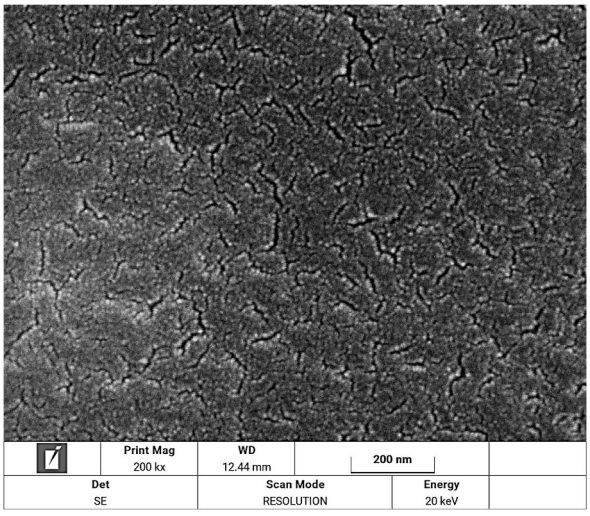

Fig. 6. FE-Scanning electron microscope image of optimized nano-niosomes.

4. Discussion

Our research has two separate but related parts. In the first part, after extracting the essential oil from the seed of *Bunium persicum*, the chemical composition of the essential oil was determined using GC-Mass. In this part, the phytochemical investigation confirmed the presence of a number chemical compounds in *Bunium persicum* essential oil, each of which has specific biological characteristics. Phytochemical investigation confirmed the presence of bioactive compounds such as Limonene, Camphene, Para-cymene, γ-Terpinene and Cuminic aldehyde in *Bunium persicum* essential oil, which have high anti-parasitic potential. In 2022 Bailén et al. reported that Camphene, Para-cymene, γ-Terpinene have anti-Trichomonas gallinae effect [36]. In 2020, Santana et al. showed that Para-cymene

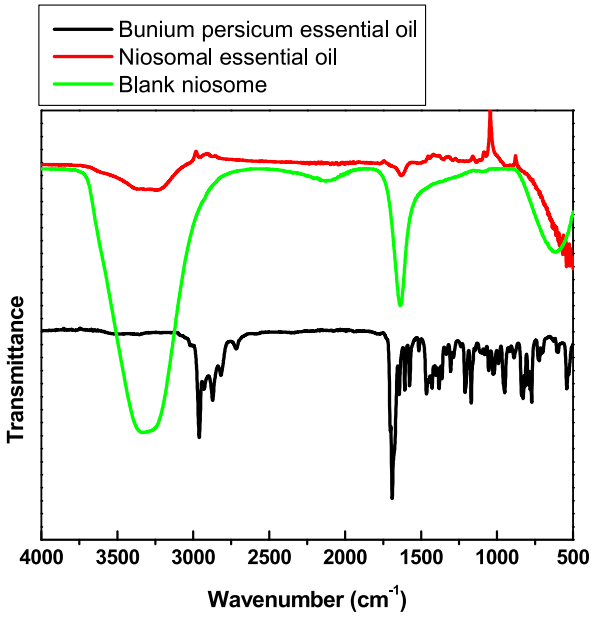

Fig. 7. FTIR spectra of Bunium persicum essential oil, blank niosome (niosome system without essential oil) and niosomal essential oil (niosome system with essential oil).

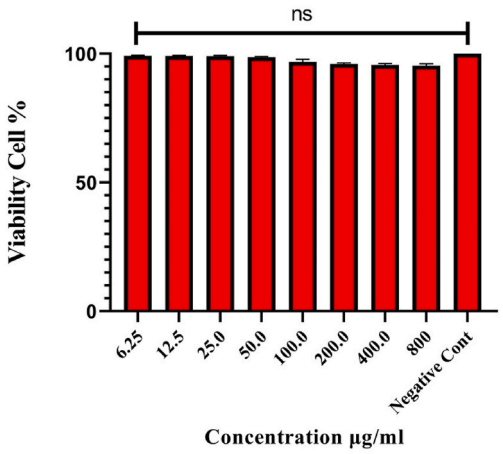

Fig. 8. Toxicity of nanoparticles containing *Bunium persicum* essential oil obtained from optimal formulation (F5-PEG) on HFF cell line.

has anti-leishmania effects [37]. Also, Arruda et al., in 2009 confirmed the antiparasitic activity of Limonene [38]. Researches that investigated the phytochemicals of *Bunium persicum* essential oil have reported chemical compounds that are similar to the compounds identified in the present study. For example, In 2023, Siyadatpanah et al. reported chemical compounds such as Camphene, Para-cymene, 1-limonene, Trans-Decalone and γ-Terpinene in *Bunium persicum* essential oil, which are very similar to the results of the present study in terms of type and percentage of compounds [1]. This similarity can be due to the fact that *Bunium persicum* was collected from the same area in both studies. In 2022, Prandelli et al. confirmed that *Bunium persicum* essential oil is contains γ-terpinene (35.8 %), cumin aldehyde (16.6 %), γ-terpinen-7-al (14.0 %) and α-terpinen-7-al(11.7 %) [39]. In 2008, Shahsavari et al. reported that *Bunium persicum* seed essential oil contains chemical compounds such as caryophyllene (27.81 %), γ-terpinene (15.19 %) and cuminyl acetate (14.67 %) [40]. Despite the similarity between the results of the current research and Prandelli's research and Shahsavari's research, some compounds identified in the current research are different from Prandelli's research and Shahsavari's research. This difference can be due to the fact that the type and percentage of compounds in the essential oil depend on various factors such as the soil and the harvest season of the plant, and with the change of each of these factors, the compounds in the essential oil can change [41]. The second part of the current research led to an optimized Niosome formulation that has a high potential for the delivery of *Bunium persicum* essential oil in the acidity and temperature of the living environment of *Trichomonas vaginalis*. In this study, the optimized nano niosomes containing *Bunium persicum* essential oil have a particle size of 159.73 nm, a negative zeta potential of −25.1

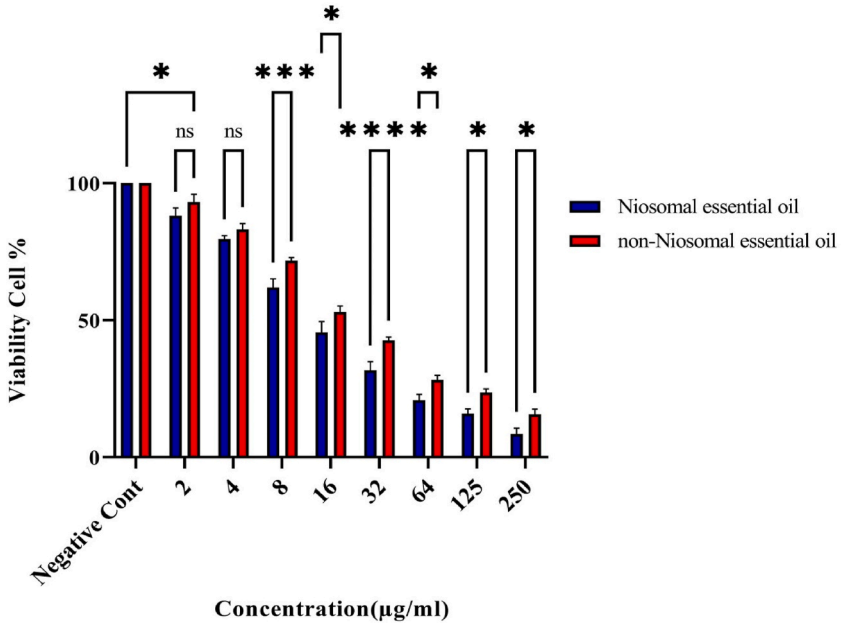

Fig. 9. Comparison of toxicity of pegylated niosomal system containing essential oil (F5-PEG) and free essential oil on *Trichomonas vaginalis* (Significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns = non-significant).

Table 4
The encapsulation efficiency (EE%), size and zeta potential of niosomal system particles during 120 days.

Days parameters	EE%	Size (nm)	Zeta potential (mV)
After 30 days	57.15 ± 1.63	161.12	−23.1
After 60 days	53.59 ± 2.22	164.2	−21.5
After 90 days	48.56 ± 1.94	165.78	−19.3
After 120 days	41.72 ± 2.9	168.10	−18.1

mV and an encapsulation efficiency of 63.11 %. These lipid nanocarriers release *Bunium persicum* essential oil slowly and continuously in the similar acidity and temperature as the environment of *Trichomonas vaginalis*. One of the most important characteristics of nanocarriers is the encapsulation efficiency. The encapsulation efficiency depends on various factors such as the type and molar percentage of the compounds that make up the nanocarrier, the type and nature of the substance loaded in the nanocarrier and the manufacturing method [42]. In the present study, among formulations F1 to F6, the lowest encapsulation efficiency is related to formulation F2 and the highest encapsulation is related to formulations F5 and F3. This difference in encapsulation efficiency can be caused by the type and percentage of compounds used in the structure of nano-niosomes obtainedfrom F1 to F6 formulations. Tween 60 and 80, which has been used in the formulation of F3 and F5, due to its longer carbon chain, creates larger nanoparticles that can increase the encapsulation efficiency. But, the nano niosomes that were made from Tween 40, have a smaller size due to the short carbon chain in Tween 40, which can reduce the encapsulation efficiency in these nanoparticles [43]. Another factor affecting the encapsulation of lipid nanocarriers is the molar percentage of cholesterol. Although the use of cholesterol increases the stability of lipid nanoparticles, but by increasing its amount in the structure of lipid nanoparticles, the encapsulation efficiency decreases. Also, cholesterol, by being in the vicinity of the hydrophobic tails of lipids that make up lipid nanocarriers, occupies the space of hydrophobic drugs and thus can reduce the encapsulation efficiency of hydrophobic drugs, including essential oils [44]. In this research, it was found that by optimizing the F5 formulation with PEG polymer, the encapsulation efficiency of the optimized formulation increased, which is consistent with the results of Sasani et al., in 2018 and the results of Afereydoon et al., in 2022 [45,46]. Although some studies show that optimizing nanoparticles with PEG polymer reduces the encapsulation efficiency. For example, in 2019 Shahi et al. reported that the optimization of niosomes with PEG polymer slightly reduces the encapsulation efficiency of nano niosomes [47]. In the present study, the release of *Bunium persicum* essential oil from nano niosomes was investigated at the same temperature and pH as the living environment of *Trichomonas vaginalis*. The release of essential oil in all formulations is slow and continuous. The essential oil release pattern from the optimized formulation shows that in the early hours, the essential oil release rate is high, but later (with the passage of time), the essential oil release rate slows down. Parnian et al., in 2020 and Talei Ardakani et al., in 2020 have reported the slow and continuous release of herbal compounds from nano niosomes in their research [48,49]. It was also found that by using PEG polymer in the structure of F5 formulation and optimizing it, the release of essential oil from this optimized formulation is reduced. It seems that the PEG polymer, by being placed on the surface of the nano niosome, creates an obstacle for the release of the

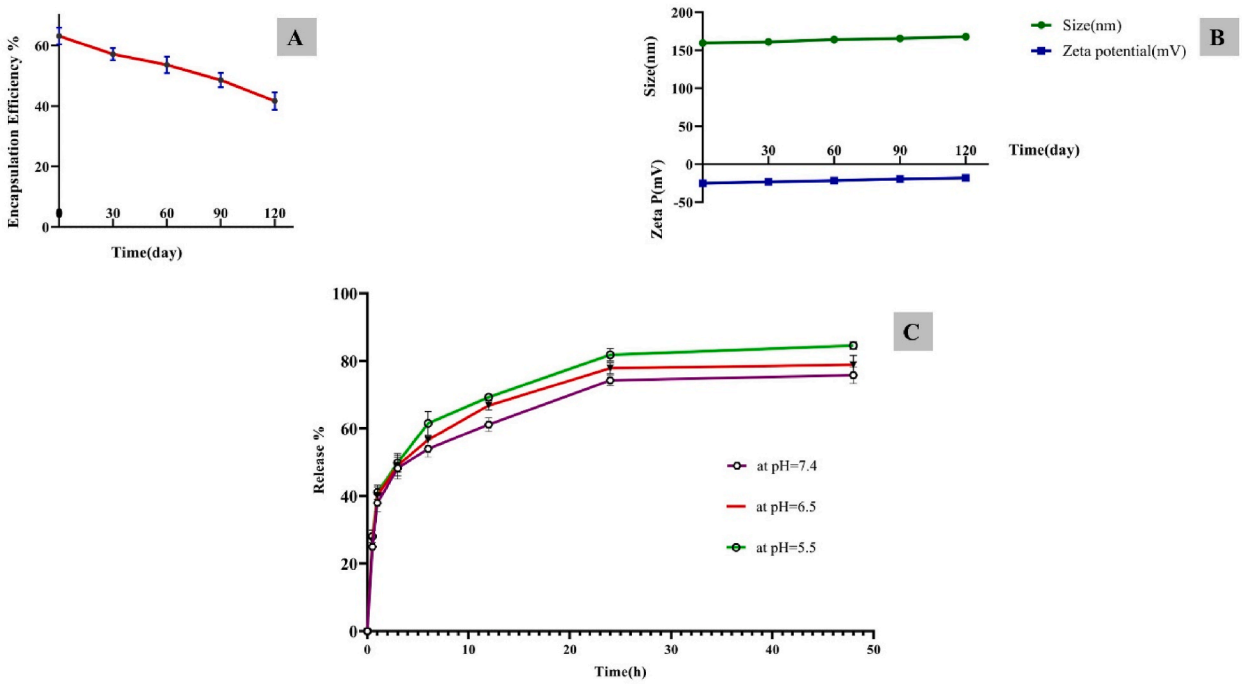

Fig. 10. Stability study of optimum formula at 4 °C for 120 days. (A) Change of stability of essential oil in niosome; (B) Change of vesicle size and zeta potential; (C) Essential oil release from nanoniosomes after 120 days at pH = 5.5, 6.5 and 7.4, within 48 h.

Table 5
The release of kinetic models and obtained parameters for the niosomal formula (after checking the stability).

	pH-7.4	pH = 6.5	pH = 5.5
Zero order	$R^2 = 0.645$	$R^2 = 0.651$	$R^2 = 0.656$
First order	$R^2 = 0.833$	$R^2 = 0.853$	$R^2 = 0.875$
Higuchi	$R^2 = 0.865$	$R^2 = 0.872$	$R^2 = 0.877$
Kors-peppas	$R^2 = 0.982$	$R^2 = 0.980$	$R^2 = 0.986$
	n = 0.2	n = 0.2	n = 0.2
	K = 36.45	K = 38.45	K = 39.84

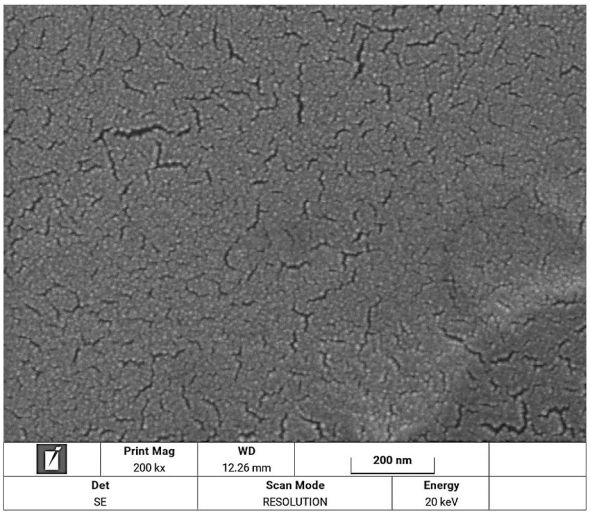

Fig. 11. FE-Scanning electron microscope image of nano-niosomes after 120 days.

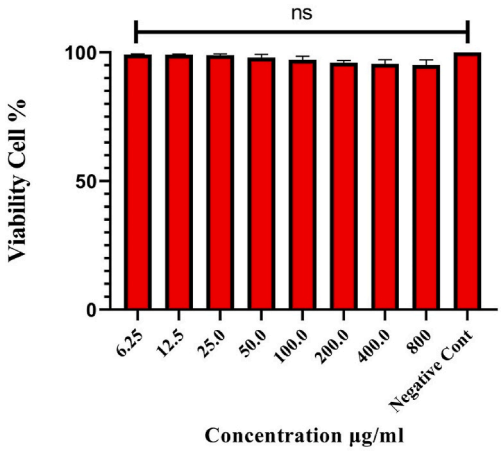

Fig. 12. Toxicity of nano-niosomes containing essential oil after 120 days on HFF cell line.

essential oil, which reduces the rate of release of the essential oil from the optimal formulation. This part of the results is consistent with Nunes' study in 2019 [50]. Mathematical modeling is an important tool in understanding drug release kinetics from nanocarriers. In the present study, zero-order, first-order, Higuchi and Korsmeyer-Pepps mathematical models were used to predict the release kinetics of Bunium persicum essential oil. Regression coefficient ($R^{2)}$ analysis in different models shows that the Korsmeyer-Pepps model with R2 close to one in all pHs has higher accuracy for predicting the release of essential oil from niosome. Also, the amount of n obtained in all releases based on this model is 0.45, which shows that the release of *Bunium persicum* essential oil follows Fick's law at all pHs [27,51]. Another characteristic of lipid nanocarriers is the zeta potential, which plays an important role in the stability of lipid nanocarriers. This physical index depends on various factors such as temperature and nano carrier components. The higher the surface charge of the particles, the greater the repulsive force between them and the accumulation and sedimentation of particles is prevented [52–56]. As a result, the stability of the lipid nano-system increases. In the present study, lipid system particles with high and negative zeta potential seem to have good stability, however, confirmation of this feature requires more laboratory studies.The results of the present study in this part are consistent with many similar studies. Also, in our study, after PEGylation of the niosomal system containing essential oil, the zeta potential of the niosomal system decreased. This reduction in surface charge can be due to the hydrodynamic resistance of PEG, which reduces the electrophoretic mobility of niosomes. Also, the presence of PEG on the surface of the niosome makes the surface charge of the niosome less exposed to measurement. This result is consistent with the results of Nunes' research [50]. Also, the size of niosomal nanoparticles in the present study is between 141.11 and 159.73 nm in all formulations. The present study shows that the size of nanoparticles increases with the increase of cholesterol. In fact, cholesterol increases the size of the vesicles by being placed between the phospholipids of the niosome bilayer [57]. Also, optimizing F5 formulation with PEG polymer increased the particle size. which can be due to the placement of PEG polymer on the surface of the nanocarrier, which increases the volume of the nanocarrier. Since *Trichomonas vaginalis* lives in the vagina, therefore, if nanocarriers are used to deal with this parasite, lipid nanoparticles will be in the vicinity of healthy body cells, so in the present study, the toxicity of nanocarriers on HFF cells was measured. Examining the toxicity of nanoparticles obtained from F1, F3 and F5 formulations on HFF cell line showed that the formulations that use Tween 60 and 80 in their structure have little toxicity on HFF cells. Arechabala et al. reported that Tween 80 has less toxicity on fibroblast cell line compared to Tween 60, which is consistent with the results of the present study [58]. The toxicity of the niosomal system containing *Bunium persicum* essential oil was more than free *Bunium persicum* essential oil within 24 h in all concentrations. This result shows that the niosome system containing essential oil has a higher potential than free essential oil in dealing with *Trichomonas vaginalis* parasite. In 2023, Siyadatpanah et al. showed that the liposomal system containing *Bunium persicum* essential oil can reduce the growth of *Trichomonas vaginalis* parasite and reported an IC50 of 14.41 µg/ml for it [1]. However, in the present study, the IC50 value was calculated as 11.01, which is lower compared to the research of Siyadatpanah et al. This reduction can be the result of the higher efficiency of niosomal nanoparticles in comparison with liposomal nanoparticles. In this research, the physicochemical stability of optimized nano-niosomes containing *Bunium persicum* oil was investigated for 120 days. During 120 days, the optimized nano-niosomes containing essential oil was kept at a temperature below 4 °C, to prevent the evaporation of the essential oil from the nano-niosomes. Examination of the optimized nano niosomes during 120 days shows that the particle size has increased slightly (8.37 nm), but the electron microscope image showed that these spherical lipid carriers have maintained their smooth surface. The zeta potential of nanoparticles during 120 days is slightly more positive (7.37 mV). The pattern of essential oil release from the optimized nanocarriers is still slow and continuous after 120 days, although the amount of essential oil release within 48 h has increased compared to the essential oil release from the optimized formulation before stability measurement. The encapsulation efficiency has decreased greatly after 120 days (21.39 %), which can be due to the volatile nature of the essential oil, which can reduce the encapsulation efficiency in the long term. With the slight changes in the physicochemical parameters of the optimized nano-niosome during 120, it seems that the optimized formulation has good stability. The results of this part of the research are consistent with the results of Akhlaghi et al.'s research in 2022 [59]. The current research has advantages and disadvantages.

Among the advantages of this research is the investigation of various formulations in order to make niosomes containing *Bunium persicum* essential oil to combat *Trichomonas vaginalis*, the study of the stability of nano niosomes containing essential oil during 120 days and the toxicity of different formulations on healthy cells. Various compounds can be used to make niosomes, including Span, which can affect the physicochemical properties of this nanocarrier (encapsulation efficiency, release, size, etc.). Therefore, not using different compounds to make niosomes and not investigating the effect of these materials on the performance of nanocarriers are among the limitations of the current research.Another limitation of the current research is the lack of investigation of different formulations for making nanoparticles. It seems that if other formulations are used for the preparation of nano nisomes in the next researches, a higher percentage of encapsulation efficiency can be achieved. Also one of the problems facing the fight against the *Trichomonas vaginalis* parasite is drug-resistant strains of this parasite. In the current research, we did not have access to the drug-resistant strain of this parasite, and the failure to investigate the effect of the nanosystem on the drug-resistant strain of the parasite is one of the limitations and shortcomings of the current research, which we suggest to other researchers in this field. Also, not investigating the release at different temperatures and pHs and not investigating the effect mechanism of nanoparticles containing essential oil on *Trichomonas vaginalis* among other limitations of current research, which are suggested to other researchers.

5. Conclusion

Considering the anti-parasitic compounds present in *Bunium persicum* essential oil, the physico-chemical properties suitable for optimized niosomes, the slow and continuous release of the essential oil at the same temperature and acidity as the environment of *Trichomonas vaginalis*, the low toxicity on healthy cells, the higher toxicity on *Trichomonas vaginalis* parasite and the suitable stability of nano-niosomes for 120 days, the optimized niosome containing *Bunium persicum* essential oil can be a suitable candidate for anti-trichomonas vaginalis therapy.

CRediT authorship contribution statement

Reza Nafisi Moghadam: Resources, Project administration. **Mohammad Majdizadeh:** Writing – original draft, Methodology, Formal analysis, Data curation. **Mohammad Golbashy:** Resources, Investigation. **Fateme Haghiralsadat:** Supervision, Project administration, Conceptualization. **Mahdie Hemati:** Visualization, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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