

Green synthesis of nano-liposomes containing *Bunium persicum* and *Trachyspermum ammi* essential oils against *Trichomonas vaginalis*

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Research

Keywords: *Trichomonas vaginalis*, *Bunium persicum*, *Trachyspermum ammi*, Essential oil, Nano-liposomes

Posted Date: December 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-32930/v5>

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Abstract

Background: *Trichomonas vaginalis*, a parasitic flagellated protozoan, is one of the main non-viral [sexually-transmitted diseases](#) worldwide. Treatment options for [trichomoniasis](#) are limited to [nitroimidazole](#) compounds. However, resistance to these drugs has been reported, which requires the development of new anti-*Trichomonas* agents that confer suitable efficacy and less toxicity.

Methods: In the present work, we assessed the effectiveness of the liposomal system containing essential oils of *Bunium persicum* and *Trachyspermum ammi* against *T. vaginalis* *in vitro*. Liposomal vesicles were prepared with phosphatidylcholine (70%) and cholesterol (30%) using the thin-film method. The essential oils of *B. persicum* and *T. ammi* were loaded into the liposomes using the inactive loading method. Liposomal vesicles were made for two plants separately. Their physicochemical features were tested using Zeta-Sizer, AFM and SEM. The anti-*Trichomonas* activity was determined after 12 and 24 hours of parasite cultures in TYI-S-33 medium.

Results: After 12 and 24 hours of administration, the IC₅₀ of the *B. persicum* essential oil nano-liposomes induced 14.41 µg/mL and 45.19 µg/mL, respectively. The IC₅₀ of *T. ammi* essential oil nano-liposomes induced 8.08 µg/mL and 25.81 µg/mL, respectively.

Conclusions: These data suggested that nano-liposomes of the essential oils of *B. persicum* and *T. ammi* may be a promising alternative to current treatments for *Trichomonas* infection.

Background

Trichomonas vaginalis (*T. vaginalis*), a flagellated parasitic protozoan, is one of the leading non-viral [sexually-transmitted infections](#) worldwide, with approximately 248 million new cases detected annually causing trichomonosis [1]. However, most infected women and men are asymptomatic, can infect the urethra and prostate in men and serious sequelae in women and more susceptible to infection by the human immunodeficiency virus (HIV) infection in men and women [2-4]. In addition, *T. vaginalis* has been associated with severe consequences, such as adverse pregnancy outcomes and preterm birth, infertility, predisposition to cervical cancer, premature rupture of membranes and pelvic inflammatory disease [5]. Moreover, this parasite can cause intellectual disability in children and respiratory and vaginal infections in neonates [6-9].

Treatment options for [trichomoniasis](#) are limited to [nitroimidazole](#) compounds. However, treatment failure occurs, mainly due to significant gastrointestinal adverse effects, which disappear after treatment. In addition, resistance to metronidazole has been reported in at least 5% of clinical cases of trichomoniasis, which could lead to higher and sometimes toxic doses in these patients. Consequently, potent new alternatives with low toxicity against *T. vaginalis* are urgently needed [10-11].

In recent years, there have been growing studies of the therapeutic potential of natural or herbal products. Medicinal plants are generally considered to be safe and have low toxicity compared to synthetic drugs

[12]. *Bunium persicum* and its derivatives have reported multi-pharmacological effects, including antimicrobial, antioxidant, anti-inflammatory, Antinociceptive, antidiabetic, antifungal and antibacterial [13-18]. *Trachyspermum ammi* has impressive biological and pharmacological properties, including antimicrobial, antihyperlipidemic, anthelmintic, antibacterial and insecticidal activities [19-23].

Liposomes, small vesicles produced by the dispersion of phospholipids in the aqueous medium, trap the aqueous medium between their closed concentric spheres of the phospholipid membranes [24-25]. Liposomes can contain hydrophilic and lipophilic drugs [26]. In addition, they are enduring and safe in clinical trials, and some formulations have been approved by the FDA [27-28]. Some methods are generally used to prepare liposomes for diagnosis and drug delivery purposes [29]. Phospholipid vesicles enhance the penetration of compounds incorporated and/or encapsulated in them.

These liposomes containing essential oils with greater permeability and concentrations, and then address concerns such as side effects, low drug solubility in water, and lack of proper drug delivery to protozoa. In the present study, the nano-liposomal lipid carriers of essential oils of *B. persicum* and *T. ammi* were used against *T. vaginalis in vitro*.

Methods

Plant collection

B. persicum and *T. ammi* were collected from the mountain Bahabad, located in the Province of Yazd, in Iran, and their species were identified and authenticated in the botanical section of the Yazd Agricultural Research Center, with the voucher numbers 1141 and 24985, respectively. A total of 100 g of seeds of *B. persicum* and *T. ammi* were harvested, dried, and mechanically powdered using an electrical blender. The whole dry of two plants was dissolved in distilled water (250 mL) overnight at room temperature using Clevenger apparatus and after 4 hours the yield was used.

Standard calibration curves of *B. persicum* and *T. ammi* (Essential Oils)

To perform the calibration curve, 1 mg/mL of *B. persicum* and *T. ammi* was prepared in methanol. Then, different dilutions of essential oils in isopropyl and PBS buffer (phosphate buffered saline) were prepared. The absorption spectra of each dilution were measured by a spectrophotometer (Carry 100 Bio Co. Varian, Victoria, Australia). The experiment was repeated three times, the standard diagram of the essential oils of *B. persicum* and *T. ammi* was drawn and then the calibration chart of essential oils of *B. persicum* and *T. ammi* was drawn in isopropyl solvent and PBS.

Preparation of liposomal systems containing essential oils of *B. persicum* and *T. ammi*

The liposomal system containing essential oils was prepared by thin-layer coating with a combination of soybean phosphatidylcholine (70%), and cholesterol (30%). Soybean phosphatidylcholine, cholesterol and essential oil were dissolved in a chloroform solvent at 45°C on a rotary (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) and dried under vacuum. Hydration was then

performed by adding sterile distilled water for one hour at 55°C. The prepared nano-liposomes were then reduced in size using a bath sonicator for 40 minutes. At the end of the mixture, the size reduction was filtered using 0.45 and 0.20 µm filters to homogenize the constituent particles.

Determination of essential oil loading percentage in liposomal systems

Liposomes containing essential oils were inserted into the dialysis bag after size reduction and filtration and incubated with distilled water for 4 hours at 4°C to remove the free and unincorporated essential oil. Liposomes were mixed with 1:20 isopropyl to break the lipid wall around the essential oil and release the essential oil. The amount of essential oil absorbed in the liposomal system was determined by a wavelength spectrophotometer. Finally, using the following relationship, the percentage of *B. persicum* and *T. ammi* load of essential oils in liposomal systems was determined separately.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{essential oil in liposome (mg)}}{\text{Total of essential oil (mg)}} \times 100$$

Essential oils release from liposomal systems

The release of essential oils from liposome system was performed by the dialysis bag method. In this method, liposomes containing the essential oils were poured into the dialysis bag and placed in the vicinity of the PBS buffer at 35°C and pH = 6 for 24 hours. The absorbance was read by spectrophotometer (570 nm) and based on this, the release chart of liposome essential oils was drawn. All steps were performed separately to release from the liposome system containing *B. persicum* and *T. ammi*. The release results were analysed according to the standard deviations.

Size and zeta potential characterization of essential oils in liposomal systems

The particle size distribution range, as well as particle size peak, was determined using Dynamic Light Scattering (DLS), by nanosizer (Brookhaven Instruments Corp, NY, USA). Nano-liposomes were measured at an angle of 90 degrees and laser light irradiation at 657 nm at 25°C was used. The sample was prepared diluted to 0.1 mg/mL. Measurements were performed immediately after preparation. Samples were measured 3 times and each time for 30 seconds. In addition, surface charges (zeta potential) of nano-liposomes containing essential oils were measured by Zeta Sizer (Brookhaven Instruments Corp, NY, USA).

Morphology of liposomal systems containing essential oils

The liposomal system was imaged using an Atomic Force Microscope (AFM: Nano wizard II, JPK instruments, Berlin, Germany) to confirm the formation of essential oil-bearing nano-liposomes containing essential oil. In addition, 25 µL of liposome sample was poured onto a slide and air dried. Samples were then coated with gold for a few seconds to be conductive. At the end, the surface

morphology of the nano-carriers (roughness, shape, smoothing and mass) was investigated using a Scanning Electron Microscope (SEM: EM3200, KYKY Technology Development Ltd, Beijing, China).

Cytotoxicity studies by MTT assay

The cytotoxicity of the empty liposomal system was determined by the MTT assay. To measure toxicity, cells were cultured separately at 1×10^4 cells in a 96-well plate for 24 hours. Healthy human foreskin fibroblast (HFF) cells were then treated with the same volume of fresh culture medium. Then, HFF fibroblasts were cultured in four replicates at 100 and 10 $\mu\text{g/mL}$, respectively, and incubated again for 48 hours. Then, 20 μL of MTT solution at a concentration of 5 mg/mL were added to each well and incubated for 3 hours. The supernatant was then removed and 150 μL of DMSO was added to dissolve the Formosan crystals. At each step, centrifugation was performed to remove the liquid. The absorbance was recorded at 570 nm using a spectrophotometer. Finally, cell viability was calculated according to the relationship below:

$$\frac{\text{Mean optical absorption in the test group} - \text{Average light absorption in culture medium}}{\text{Mean optical absorption in control group} - \text{Average light absorption in culture medium}}$$

Determination of IC_{50} nano-liposomes containing essential oil on *T. vaginalis*

The strain of *T. vaginalis* was isolated from the vaginal discharge of a 34-year-old woman with a confirmed diagnosis of trichomoniasis. This study was approved by the ethical committee of the University of Medical Sciences of Isfahan, Isfahan Province, Iran (Ref. No. 1396.851) to obtain human biological samples. The parasite isolate was cultured in TYI-S-33 medium and incubated at 37°C until reaching the number of parasites in the logarithmic phase. Smears were prepared to determine the number of parasites. The parasitic strain of the logarithmic phase (10^5 parasites) was then exposed to different concentrations of *B. persicum* and *T. ammi* nano-liposomes at a concentration of 100,000 per mL (grouped according to Table 1).

All groups were kept at 37°C and after 12 and 24 hours the number of live parasites was counted by Trypan blue using a Neubauer smear and light microscope (Haemocytometer method). Growth inhibition was calculated for each of the different concentrations (1.95- 1000 $\mu\text{g/mL}$) of liposome-containing essential oils in the parasite strain by the formula $\text{GI} = (a-b/a)$. Where **a**: the number of live parasites in the negative control sample, **b**: the number of live parasites in the sample containing *B. persicum* and *T. ammi* nanoliposomes. Then, SigmaPlot™ 13 software was used to determine IC_{50} (50% inhibitory concentration) of the above nano-liposomes. It should be noted that all experiments were performed in three replicates separately for liposomal nano-carriers containing essential oils of *B. persicum* and *T. ammi* and the results were shown in an average level. Selectivity Index (SI) is obtained from the IC_{50} value of liposomal nano-carriers containing essential oils of *B. persicum* and *T. ammi* against HFF cells

divided by the IC₅₀ value of *T. vaginalis*. Selective activities of the compounds were calculated as follows:

Selectivity Index (SI) = IC₅₀ Cells HFF/IC₅₀ Cells *T. vaginalis*

Cellular uptake study

A total of 10⁵ HFF cells in 6-well plates were grown in a monolayer for 24 hours. Then, the cells with the optimal nano-liposomes formula contained DIL color without essential oil. For the fluorescence of the Newsome system and, to a large extent, 0.1% M in the organic phase to dry the structure, the additives were followed by the steps of the thin film method, as described by Alemi A et al, 2018 [30]. Subsequently, fluorescent colored nano-liposomes attached to the DIL. These were incubated with essential oils for 3 hours and washed with PBS. Subsequently, the 95% alcoholic solution was stabilized and the nuclei were stained with DAPI (1 mg/mL) for 15 minutes. The cells were harvested and then examined under a fluorescent microscopy (Olympus, Okayama-shi Okayama, Japan).

Statistical analysis

In this study, all data were recorded, edited, and entered in the Excel software to plot the results which were reported as mean and standard deviation. Meanwhile, the SigmaPlot™ 13 software (Systat Software Inc., San Jose, CA, USA) was also used to calculate IC₅₀.

Results

Essential oils spectroscopy

For spectrophotometric data, the calibration curves of the essential oils containing *B. persicum* and *T. ammi* were plotted in the isopropyl solvent and PBS. These were shown as $y = 0.0179x + 0.1686$ and $y = 0.025x - 0.6296$ with a correlation coefficient (r^2) of 0.9962 and 0.9947 for isopropyl solvent and PBS, respectively (Fig. 1). The line diagram equation in the calibration of essential oil containing *T. ammi* in isopropyl solvent and PBS was $y = 4.278x + 0.1473$ and $y = 3.6511x + 0.0362$, respectively. The regression coefficient (R^2) of the essential oil containing *T. ammi* in the isopropyl solvent and PBS was 0.9985 and 0.9996, respectively (Fig. 1). Figure 1 shows the release results of the essential oils containing *B. persicum* and *T. ammi* based on the standard deviations.

The loading rate of essential oils in the nano-liposomal systems

Based on the calibration diagram of the essential oils containing *B. persicum* and *T. ammi* in isopropyl (Fig. 1) and the loading rate of both essential oils in the liposomal systems, were 51.64 ± 1.24 and $40.12 \pm 2.71\%$, respectively.

Patterns of release of essential oils from the nano-liposomal systems

The release charts for essential oils containing *B. persicum* and *T. ammi* from the liposomal system were plotted during 24 hours at 35°C and pH = 6 based on the calibration chart. Examination of the essential oil release pattern of the liposomal system (Table 2 and 3) shows that the release of the essential oil in the first hours (at 6 hours after release) and the sustained release of the essential oil in the last hours (interval 12 within 24 hours after release), nano-liposomal essential oils containing *B. persicum* and *T. ammi* were present in this study. In addition, the release charts for the two types of essential oils were removed from both nano-liposomal systems in 24 hours (100% release in 24 hours). The release results were presented in Figure 1, Tables 2 and 3 with standard deviations.

Characterization of the size and zeta potential of essential oils in the nano-liposomal systems

The DLS results show that the particle size in the nano-liposomal system of essential oils containing *B. persicum* and *T. ammi*, 121 nm and 111 nm, respectively (Figures 2 and 3). Figures 2 and 3 show the size of the liposomal system containing essential oil of *B. Persicum* and *T. Ammi*. In addition, the surface charge (zeta potential) of the nano-liposomal systems of essential oils containing *B. persicum* and *T. ammi* was calculated to be -16.7 mV and -9 mV, respectively (Figures 4 and 5). Figures 4 and 5 show the zeta potential of the liposomal system containing essential oil of *B. Persicum* and *T. Ammi*.

The present study led to the production in the nano-liposomal systems of essential oils containing *B. persicum* and *T. ammi*. The results showed that the load of essential oils containing *B. persicum* and *T. ammi* in the nano-liposomal systems was 51.64 ± 1.24 and $40.12 \pm 2.71\%$, respectively.

The particle size and zeta potential were also calculated for the nano-liposomal systems containing *T. ammi* were 111 nm and -9 mV, whereas for *B. persicum* they were 121 nm and -16.7 mV, respectively. The release of essential oils of *B. persicum* and *T. ammi* from the liposomal system was slow in 24 hours. The essential oils from both nano-liposomal systems were released at the end of this period (24 hours).

Nano-liposomal systems containing essential oils morphology

The morphology on the nano-liposomal systems of both essential oils containing *B. persicum* and *T. ammi* was investigated by the Atomic Force Microscope (AFM), Scanning Electron Microscope (SEM), and the formation of liposome was confirmed (Figures 6 and 7). Figure 6 shows the AFM photograph of liposomal system containing essential oil of *B. Persicum* and *T. ammi*. The SEM image shows that the morphology of the constituent particles are liposome systems containing essential oil, are spherical with smooth surface and average particle size from 25 to 40 nm. Figure 7 shows the SEM photograph of liposomal system containing essential oil of *B. Persicum* and *T. ammi*.

In addition to electron microscopy images, it also confirms the formation of liposomal systems, spherical morphology and the appropriate constituent particles of liposomal systems. Cellular studies also showed that the nano-liposomal systems had low toxicity for HFF cells, and the nano-liposomal systems of essential oils containing *T. ammi* and *B. persicum* for 24 and 48 hours showed toxicity for the parasite *T. vaginalis* and were further prevented its growth.

Cytotoxicity studies by MTT assay on Human foreskin fibroblasts (HFF) cell line

The evaluation of the effect of the essential oil-free liposomal system on human foreskin fibroblast (HFF) cells showed that the viability of these cells in the presence of 10 and 100 µg/mL of an empty liposomal system is 96.7% and 97.2%, respectively. Thus, the empty liposomal system has little toxicity to HFF cell lines.

Nowadays, the use of herbal compounds for therapeutic purposes has been widespread and numerous studies have been conducted on the effects of herbal compounds on health and treatment. In the present study, after the development of liposomal systems containing essential oils of *B. persicum* and *T. ammi*, the cytotoxic effects of the vacuolar system on the HFF cell line and the cytotoxic effects of essential oil systems in *T. vaginalis* were investigated, with minor effect on the HFF cell line.

IC₅₀ of nano-liposomes containing the essential oil against *T. vaginalis*

The IC₅₀ of *B. persicum*-containing liposome essential oil was calculated to be 45.19 µg/mL and 14.41 µg/mL in *T. vaginalis* after 12 and 24 hours, respectively. The IC₅₀ value of the nano-carrier containing *T. ammi* essential oil was determined after 12 and 24 hours against the parasite, *T. vaginalis*, at 25.81 µg/mL and 8.08 µg/mL, respectively (Fig. 8 and 9). Figures 8 and 9 show the IC₅₀ content of nano-liposomes containing essential oil of *B. persicum* and *T. ammi* against *T. vaginalis* after 12 and 24 hour incubation. The value of SI (Selectivity Index) were 3.1 for *T. ammi* and 2.49 *B. persicum*.

Cellular Uptake Study

Highly successful nano-liposomes cell extract of containing essential oils without fluorescent dyes DIL was synthesized by fluorescent microscopy. According to the Fig. 10, the intensity of green fluorescence in healthy HFF skin cells indicates the cellular uptake of nano-liposomes essential oils, nano-liposomes are well absorbed by the cell. Figure 10 shows the fluorescent microscopic images of cellular uptake of nano-liposomes containing essential oils of *T. ammi* and *B. persicum* results. Core-shaped with DAPI color (blue) and colored DIL nanoliposomes (green) were painted.

Discussion

So far, previous studies have been performed on the production of lipid systems containing drugs and essential oils, each of which investigates different physicochemical factors of lipid systems, including loading efficiency, drug release pattern, particle size and surface charge (zeta potential) [31-32].

This physicochemical index depends on several factors, including the size of the constituent system, the type and molar percentage of phospholipids used in the structure of the liposomal system, the molar percentage of cholesterol used in the structure of the liposomal system and the nature of the loaded material.

In the present study, the amount of essential oils containing *B. persicum* and *T. ammi* in the liposomal system was measured as 40.12 ± 2.71 and $51.64 \pm 1.24\%$, respectively. Other studies have yielded similar results, for example, Majdizadeh et al., 2018, reported loading of essential oil of *Mentha piperita* by 61.38% [26]. In addition, Haghirsadat et al, 2016 reported the loading rate of essential oil of *T. ammi* in the liposomal system as 35.6 ± 7.4 [33]. Other features studied in liposomal systems are the patterns of drug release. The present study showed that the release of essential oils of *B. persicum* and *T. ammi* from liposomal systems was slow in 24 hours.

The pattern of release of these essential oils from the liposomal systems shows that, due to the high concentration of essential oils between the liposomal systems and the buffer that surrounds them, the release of essential oils from the system was explosive in the first hours and decreased over with time. This difference in concentration decreased the release of the essential oil and caused the slope of its release chart to tilt to zero [34].

Another important feature evaluated in this study was the level of superficial charge of the liposomal systems, which is one of the factors that influence the stability of the liposome. There is a direct relationship between the surface charge and the chance/stability of the liposomal systems. Therefore, the greater the surface load of the liposomal system, the greater the chance that these liposomal systems will form and aggregate, increasing their stability [35-37].

vaginalis in vitro. In this study, extracts from *Micania cordifolia* and *Scutia buafulia* from the Asteraceae family and *Lobalia neurolarea* from Rhamnaceae family had the greatest effect on *T. vaginalis* [34]. Ziaiye et al. (2006) showed that the methanolic extract of *Zataria multiflora* Boiss, *Myrtus communis* and *Artemisia aucheri* Boiss had similar effects on the growth of *T. vaginalis* [35].

Wachter et al. (2014) investigated the effects of different concentrations of curcumin on *T. vaginalis* and reported that curcumin had an anti-*Trichomonas* effect at all concentrations [38]. Jabari et al. (2015) investigated the effects of *Chaerophyllum macropodium* extract on *T. vaginalis* and reported an inhibitory effect at all concentrations (2 to 150 mg/mL). As the concentration of the extract increased, the inhibitory effect also increased [39]. Vazini et al. (2017) investigated the effect of the nano-emulsion of the extract of *Micana cordifolia* on the growth of *T. vaginalis* and reported that 1000 ppm of nano-emulsion extract resulted in the elimination of 100% of this parasite [40]. The results of the Vazini et al. (2017), as in the present study, confirm the anti-*Trichomonas* activity of nano-liposomes containing essential oils. Fakhrieh-Kashan et al. (2017) evaluated the combined anti-*Trichomonas* effects of *Ginger officinale* and *Verbascum thapsus* extracts and reported that these extracts could increase apoptosis in *T. vaginalis* [41].

Majdizadeh et al. (2018) developed a liposomal system containing peppermint essential oil in a thin layer with a particle size of 247 nm, a zeta potential of -34.54, a loading efficiency of 61.38% and a slow release at 62 hours [28]. It seems that similarities in the construction of the lipid system, the type of materials used in the lipid system and the nature of the loaded material are some of the factors that supports the results of the present study. In a study of the effect of rosemary on *T. vaginalis*, Saeidi et al.

(2012) reported inhibitory effects on the growth of this parasite at concentrations of 0.0001 and 0.0002 mg/mL [42]. Recently, Mohammadi *et al.* (2019) developed a liposomal system that uses ginger essential oil to investigate the antifungal effects of this system and is related to the essential oil containing 95 nm liposomal system, having significant antifungal effects on *Aspergillus parasiticus* [43]. This similar finding with our study confirms the antimicrobial activity of lipid systems containing essential oils.

In this study, DIL-liposome solution treatment group, a very weak green signal was detected in the cell cytoplasm, whereas a relatively stronger signal was observed in the *T. ammi* essence of DIL-liposome and in the DIL-liposome treatment group. A similar uptake pattern has been reported for calcein-loaded liposomes [44], as well as for hyaluronic acid-magnetic-loaded liposomes [45]. It has been reported that liposome can interact with cells by four mechanisms, including endocytosis, adsorption to the cell surface, transfer of liposomal lipids to the cell membrane and lipid-mediated fusion with the intracellular membranes, the main pathways for liposomal internalization [46, 47, 48]. Moreover, factors such as size due to aggregation, surface charge and surface affinity seem to have a major impact on nanoparticle binding to the cell membrane and subsequent cell uptake [49]. The results indicated that the cellular uptake of the nano-liposome by the skin cells was enhanced by the presence of the intensity of fluorescence signal in the skin cells.

Conclusions

The results showed significantly improvement in the co-entrapment of *B. persicum* and *T. ammi* for the liposomal systems, which had suitable stability, controlled release rate of essential oils, good surface zeta potential and suitable mean diameter (< 50 nm). The results of this study confirmed the appropriate physical and chemical properties of the liposomal of *B. persicum* and *T. ammi*, essential oils, and demonstrate for the first time of their high potential in the treatment of *Trichomonas* infection. Further studies are recommended to gain a better understanding of the mechanisms involved.

Abbreviations

T. vaginalis: *Trichomonas vaginalis*; *B. persicum*: *Bunium persicum*; *T. ammi*: *Trachyspermum ammi*; DLS: Dynamic Light Scattering; AFM: Atomic Force Microscope; SEM: scanning electron microscopy; HFF: Healthy human foreskin fibroblast.

Declarations

Acknowledgements

We thank the Project CICECO-Aveiro Institute of Materials, FCT Ref. UIDB/50011/2020 & UIDP/50011/2020 for the financial support. We also thank the staff of the research laboratory at Shahid Sadoughi University of Medical Sciences, Yazd, Iran, for their help and contributions.

Ethics approval and consent to participate

All experiments were approved by the local Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (No. 1399.150).

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Shahid Sadoughi University of Medical Sciences, Yazd, Iran; Grant number 5439 and Ethics code was IR.B.UMS.REC.1399.150.

Authors' contributions

FM, BFH and FR conceived, designed the study and conducted the laboratory work. RN and AS analyzed the data and interpreted the results. RN, VN, WM, MN, ML.P, SAH, MM and MM wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Effects of *B. persicum* and *T. ammi* nano-liposomes against *T. vaginalis*

Number of groups	TYS-I-33 medium culture group	Final concentration of <i>B. persicum</i> and <i>T. ammi</i> nano-liposomes (µg/mL)	<i>T. ammi</i> number of parasites after 12 hours	<i>T. ammi</i> number of parasites after 24 hours	<i>B.</i> <i>persicum</i> number of parasites after 12 hours	<i>B.</i> <i>persicum</i> number of parasites after 24 hours
Negative control	Containing unexposed parasites	-	10,7000	132,705	102,894	127,252
Positive control	Containing the 10 ⁵ parasites (<i>T. vaginalis</i>) and metronidazole (64 µg/mL)	-	-	-	-	-
1	Contains the 10 ⁵ parasites	1.95	103,792	120,761	94,662	106,892
2	Contains the 10 ⁵ parasites	3.9	93,091	100,857	84,373	80,169
3	Contains the 10 ⁵ parasites	7.81	81,320	84,935	68,939	55,991
4	Contains the 10 ⁵ parasites	15.62	69,553	61,044	57,621	39,448
5	Contains the 10 ⁵ parasites	31.25	60,992	35,832	47,331	20,361
6	Contains the 10 ⁵ parasites	62.5	52,436	19,906	39,099	10,184
7	Contains the 10 ⁵ parasites	125	40,667	1,327	29,839	1,273
8	Contains the 10 ⁵ parasites	250	28,896	0	17,492	0
9	Contains the 10 ⁵	500	17,125	0	5,145	0

	parasites					
10	Contains the 10 ⁵	1000	3,211	0	0	0
	parasites					

Table 2 The release of the essential oil containing *B. persicum* in PBS

int number	1	2	3	4	5	6	7	8	9	10	11	12	13
ime (hour)	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	6	12	24
elease (%)	39.17	44.42	48.47	55.16	62.39	66.99	68.42	69.09	69.32	69.56	77.79	83.61	95.25
Std	1.02	1.04	0.66	2.86	0.98	1	0.28	0.74	0.32	0.36	2.34	1.06	3.06

Table 3 The release of the essential oil containing *T. ammi* in PBS

Point number	1	2	3	4	5	6	7	8	9	10	11	12	13
Time (hour)	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	6	12	24
Release (%)	16.55	19.62	21.41	29.11	37.12	40.07	45.84	53.03	57.84	65.74	76.42	95.82	100
Std	1.06	0.4	1.3	.08	1.6	1.4	0.8	1.4	1.1	2.8	2.3	1.06	3.6

Figures

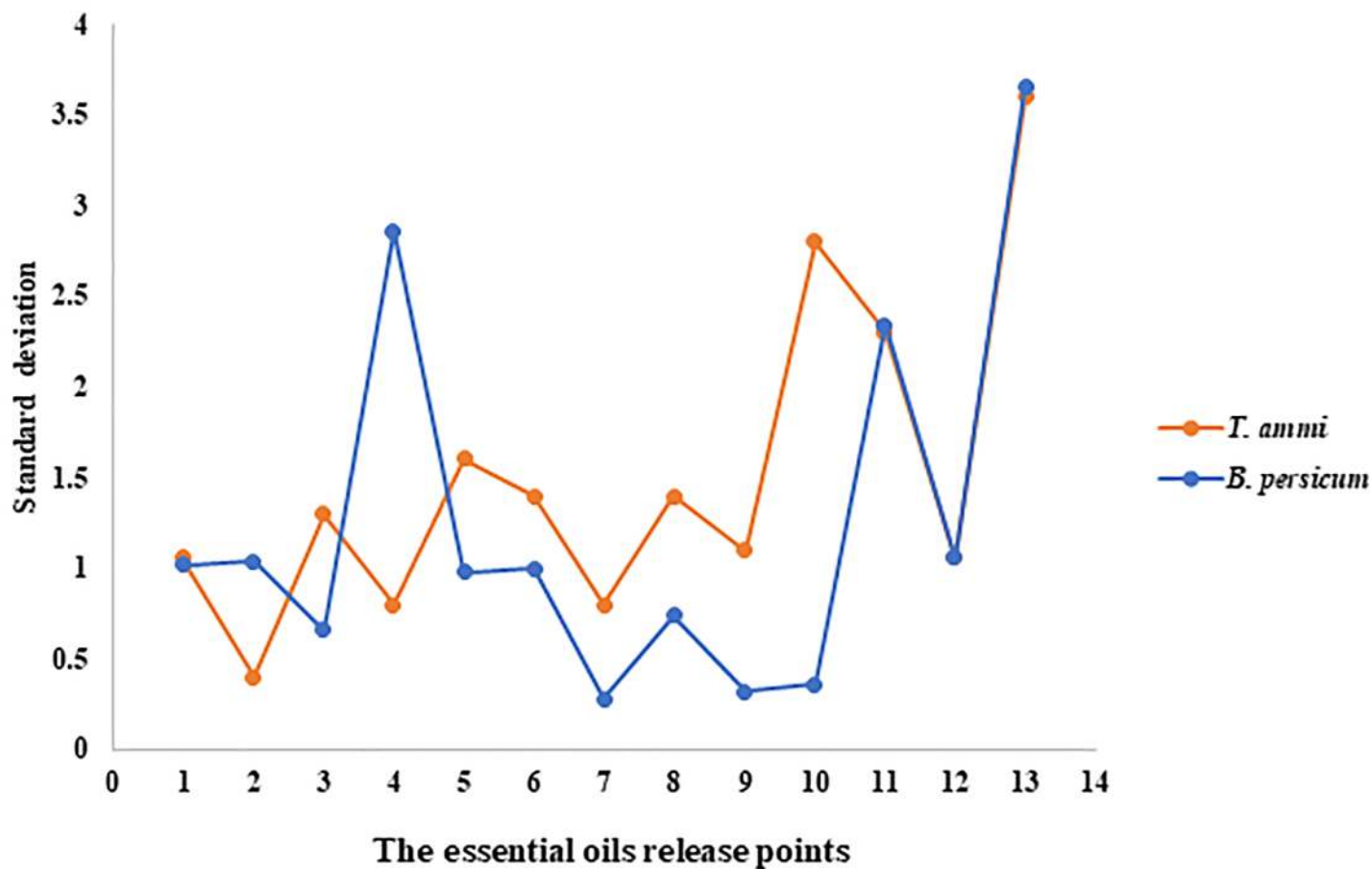


Figure 1

The release results according to the standard deviations. The release of essential oils from liposome system was investigated by measuring the absorbance at 570 nm. The data are presented as mean±SD.

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	117.3 nm	33.9 nm	99.2 nm
2	---	---	---	---
3	---	---	---	---
Total	1.00	117.3 nm	33.9 nm	99.2 nm

Cumulant Operations

Z-Average

: 145.0 nm

PI

: 0.144

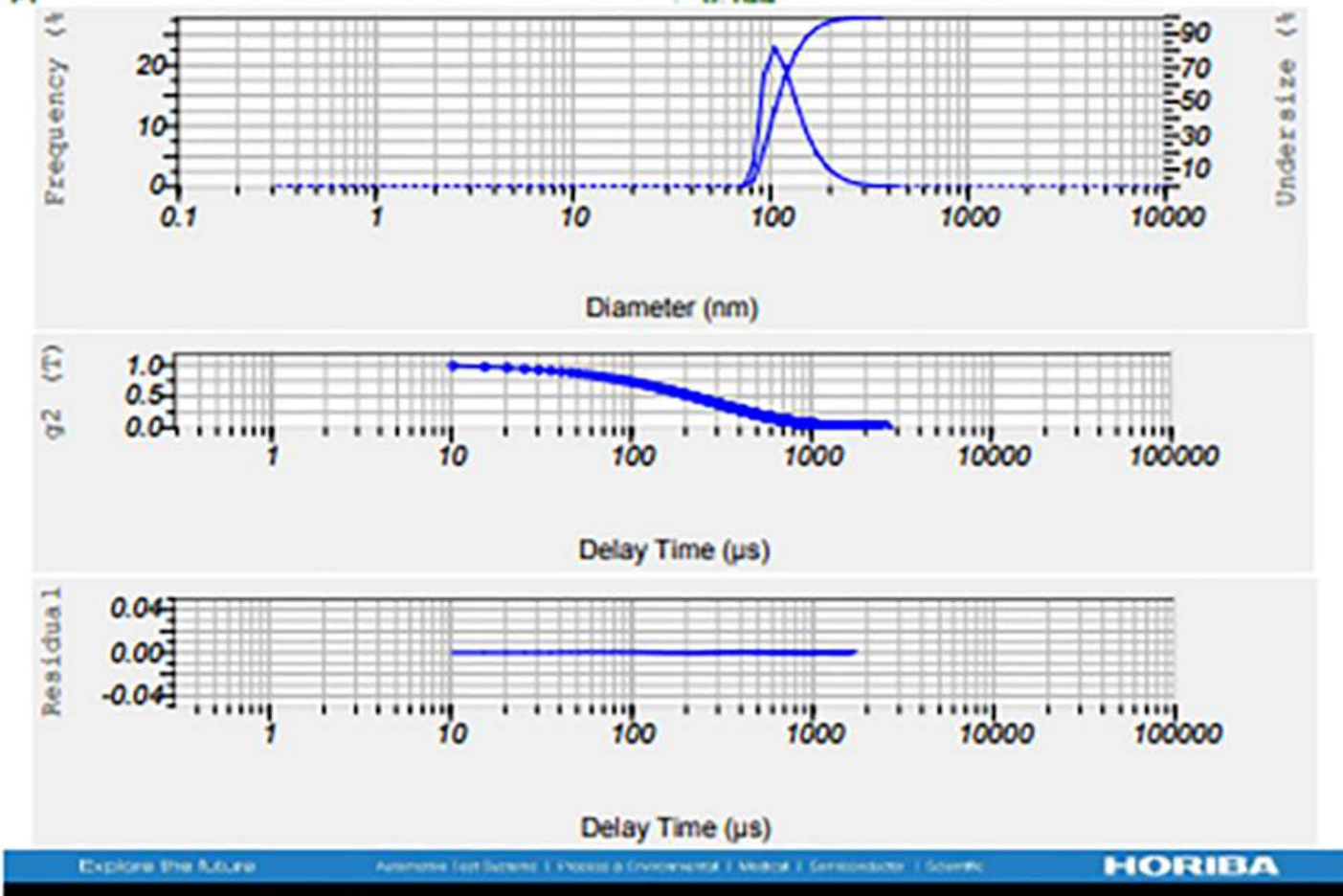


Figure 2

Size of the liposomal system containing essential oil of *B. persicum*. The particle size distribution range, as well as particle size peak, was determined using Dynamic Light Scattering by nanosizer. Nano-liposomes were measured at an angle of 90 degrees and laser light irradiation at 657 nm at 25°C was used. The data are presented as mean $\pm$ SD.

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	101.0 nm	19.6 nm	90.6 nm
2	---	---	---	---
3	---	---	---	---
Total	1.00	101.0 nm	19.6 nm	90.6 nm

Cumulant Operations

Z-Average

: 145.0 nm

PI

: 0.144

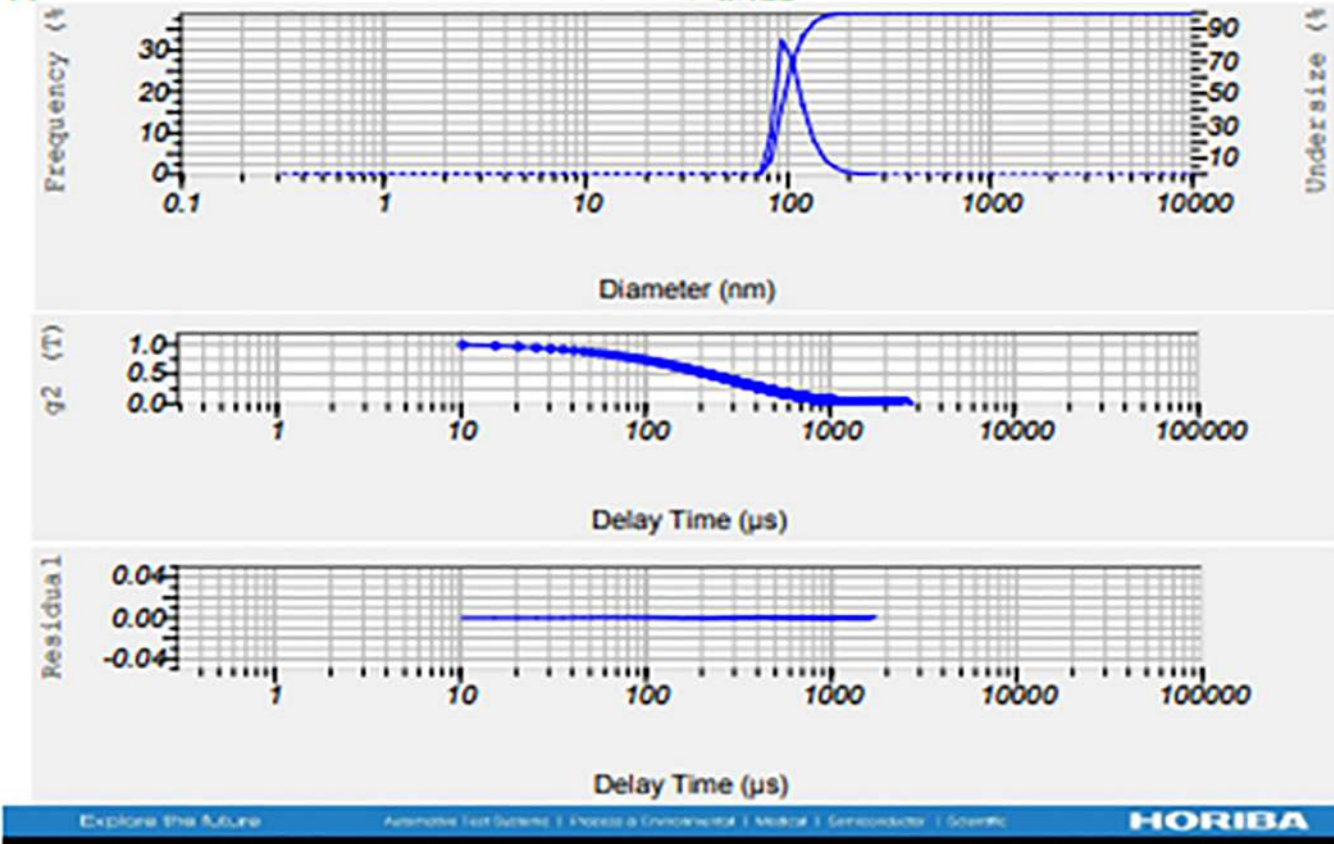


Figure 3

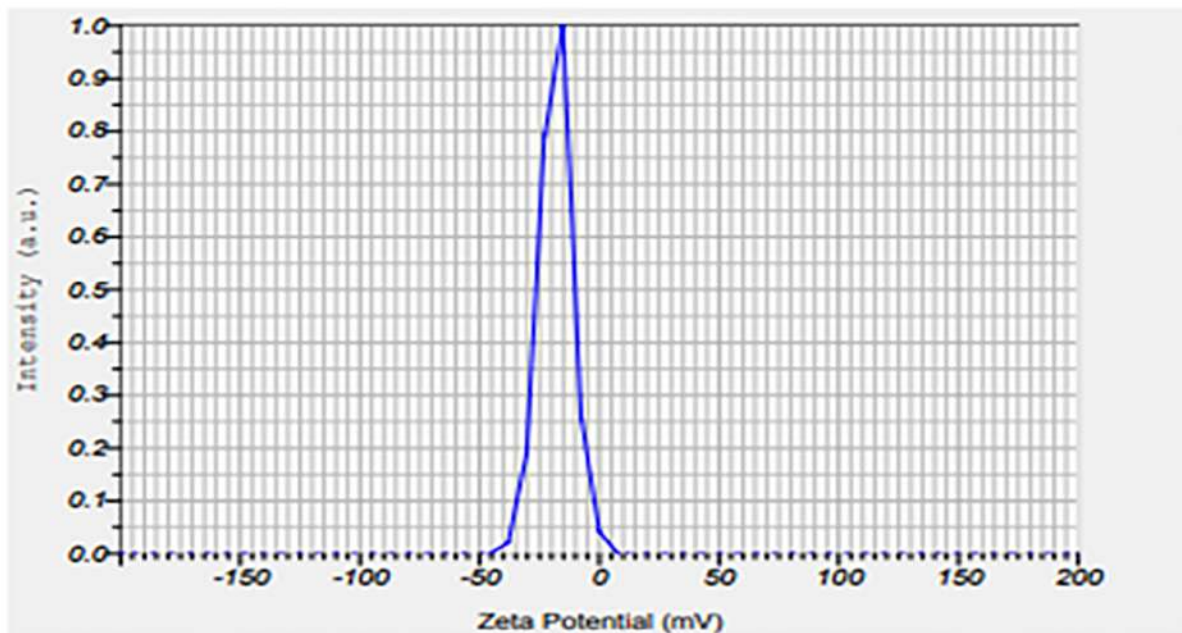
Size of the liposomal system containing essential oil of *T. ammi*. The particle size distribution range, as well as particle size peak, was determined using Dynamic Light Scattering by nanosizer. Nano-liposomes were measured at an angle of 90 degrees and laser light irradiation at 657 nm at 25°C was used. The data are presented as mean±SD.

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-18.3 mV	-0.000142 cm ² /Vs
2	— mV	— cm ² /Vs
3	— mV	— cm ² /Vs

Zeta Potential (Mean) : -18.3 mV

Electrophoretic Mobility Mean : -0.000142 cm²/Vs



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Figure 4

Zeta potential of the liposomal system containing essential oil of *B. persicum*. Zeta potential of nano-liposomes containing essential oils were measured by Zeta Sizer. Zeta potential of the nano-liposomal systems of essential oils containing *B. persicum* was -16.7 mV.

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-17.8 mV	-0.000138 cm ² /Vs
2	— mV	— cm ² /Vs
3	— mV	— cm ² /Vs

Zeta Potential (Mean) : -17.8 mV

Electrophoretic Mobility Mean : -0.000138 cm²/Vs

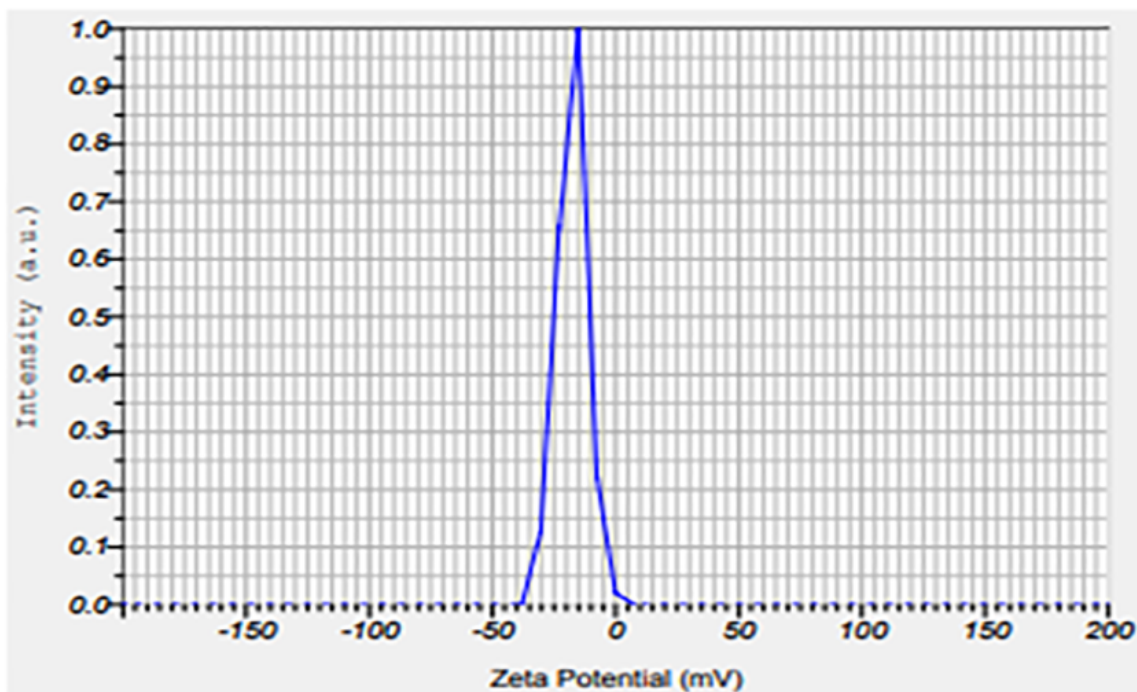


Figure 5

Zeta potential of the liposomal system containing essential oil of *T. ammi*. Zeta potential of nano-liposomes containing essential oils were measured by Zeta Sizer. Zeta potential of the nano-liposomal systems of essential oils containing *T. ammi* was -9 mV.

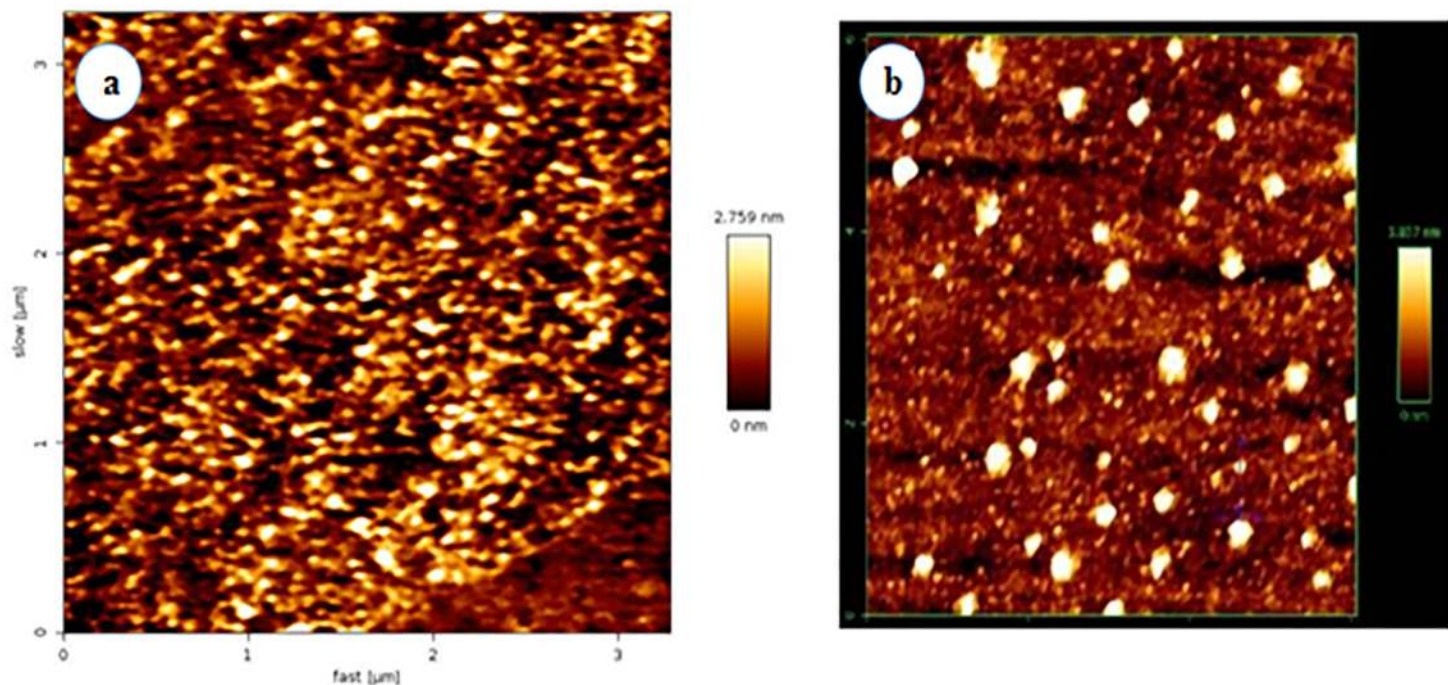


Figure 6

Atomic Force Microscope (AFM) photograph of liposomal system containing essential oil of *B. persicum* (a) and *T. ammi* (b).

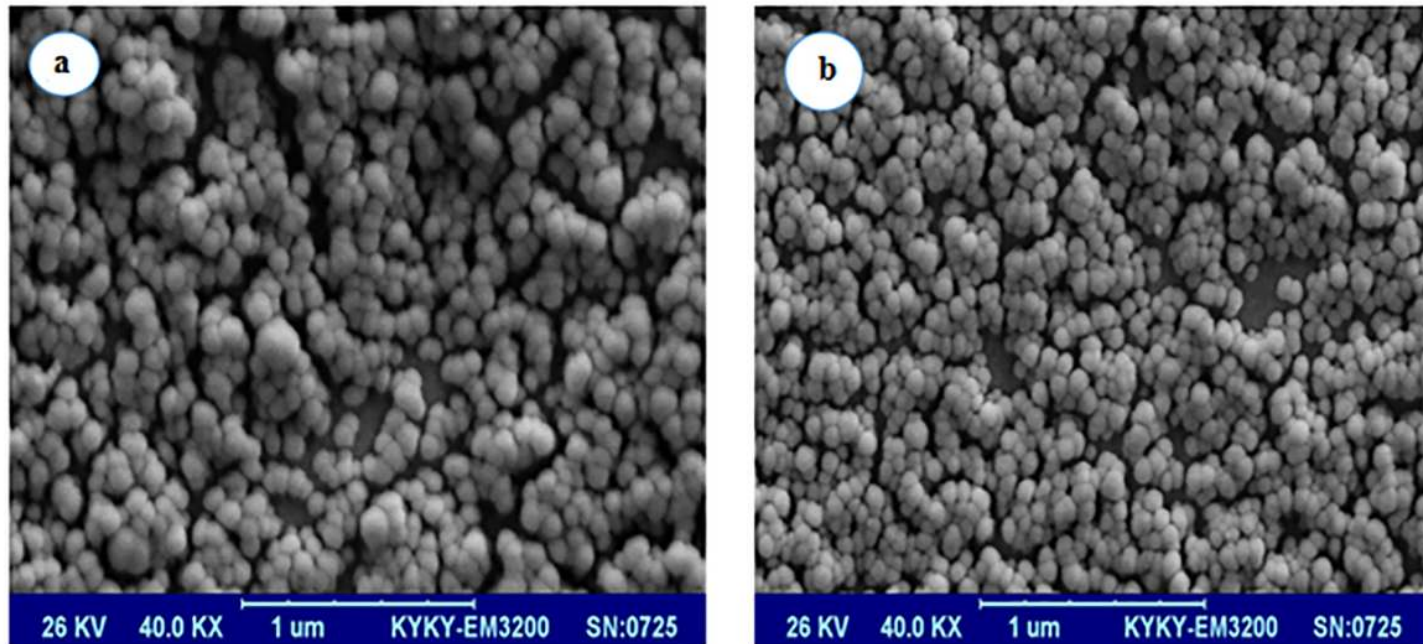


Figure 7

Scanning Electron Microscope (SEM) photograph of liposomal system containing essential oil of *B. persicum* (a); SEM photograph of liposomal system containing essential oil of *T. ammi* (b). The SEM

image shows that the morphology of the constituent particles are liposome systems containing essential oil, are spherical with smooth surface and average particle size from 25 to 40 nm.

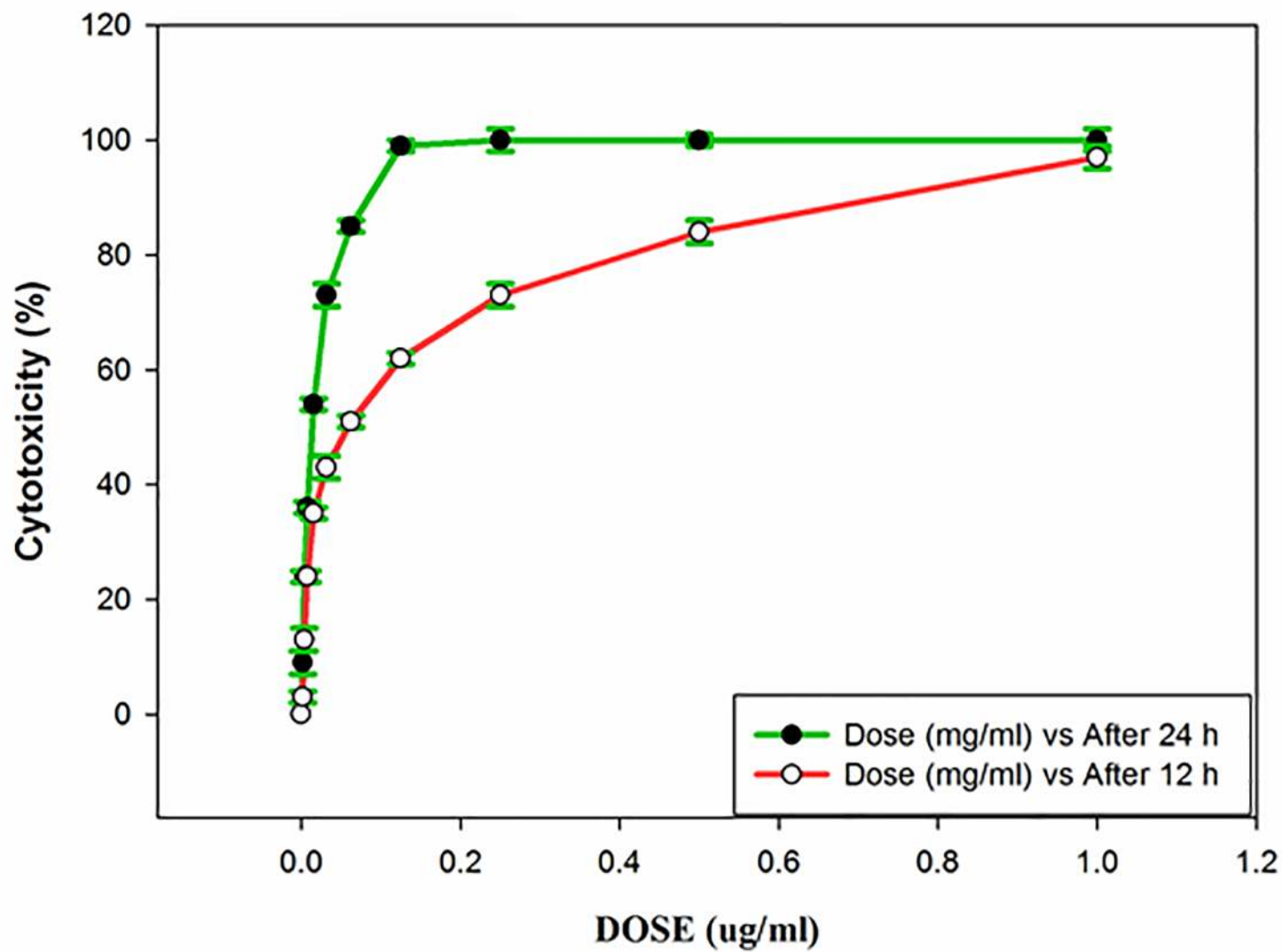


Figure 8

IC50 content of nano-liposomes containing essential oil of *B. persicum* against *T. vaginalis* after 12 and 24 h incubation. The parasitic strain of the logarithmic phase (105 parasites) was exposed to different concentrations of *B. persicum* nano-liposomes. Growth inhibition was performed by trypan blue assay, and calculated for each of the different concentrations (1.95- 1000 $\mu\text{g/mL}$) of liposome-containing essential oils. SigmaPlot™ 13 software was used to determine IC50 of the above nano-liposomes. The data are presented as mean $\pm$ SD.

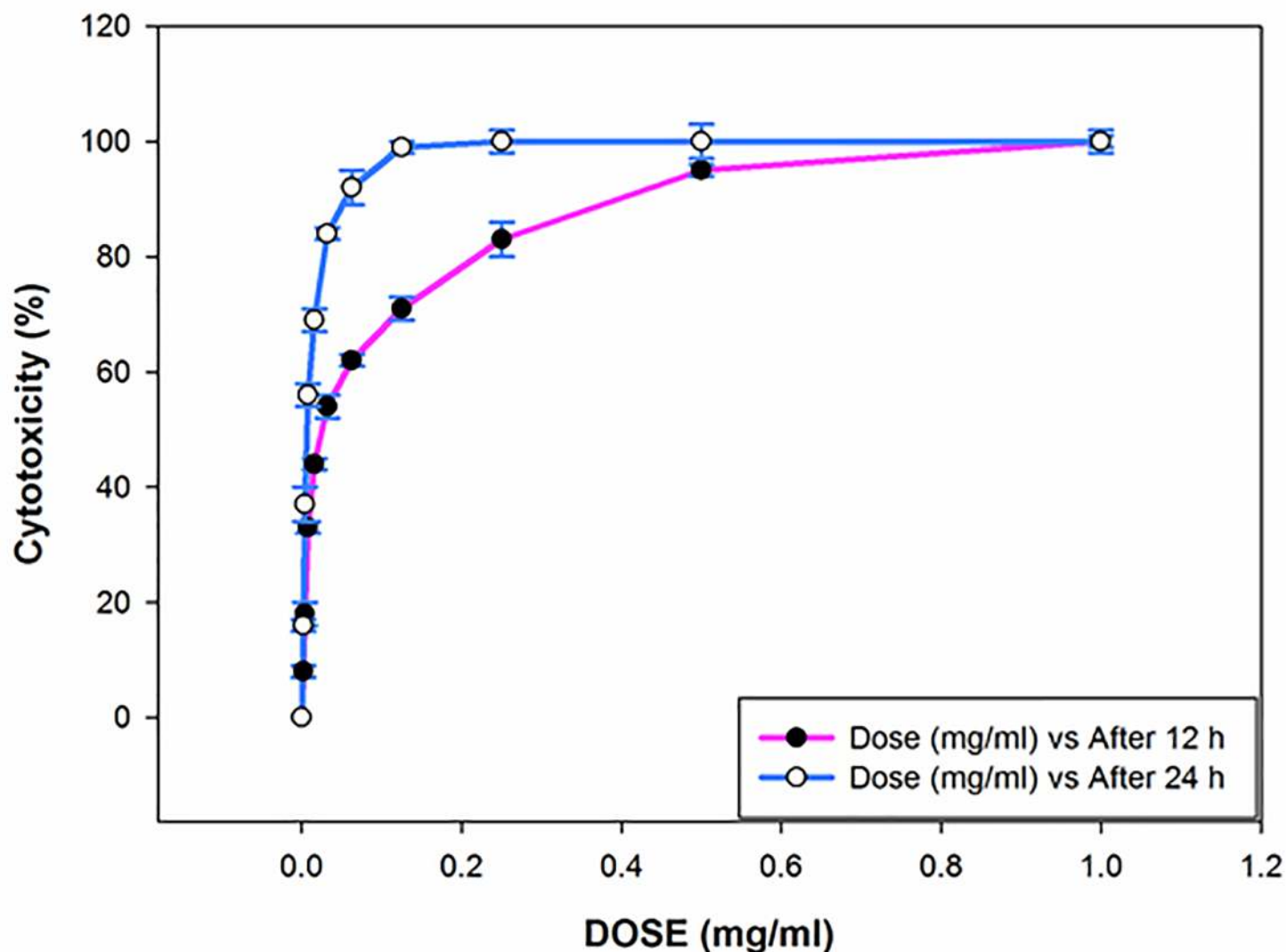


Figure 9

IC50 content of nano-liposomes containing essential oil of *T. ammi* against *T. vaginalis* after 12 and 24 h incubation. The parasitic strain of the logarithmic phase (105 parasites) was exposed to different concentrations of *T. ammi* nano-liposomes. Growth inhibition was performed by trypan blue assay, and calculated for each of the different concentrations (1.95- 1000 µg/mL) of liposome-containing essential oils. SigmaPlot™ 13 software was used to determine IC50 of the above nano-liposomes. The data are presented as mean±SD.

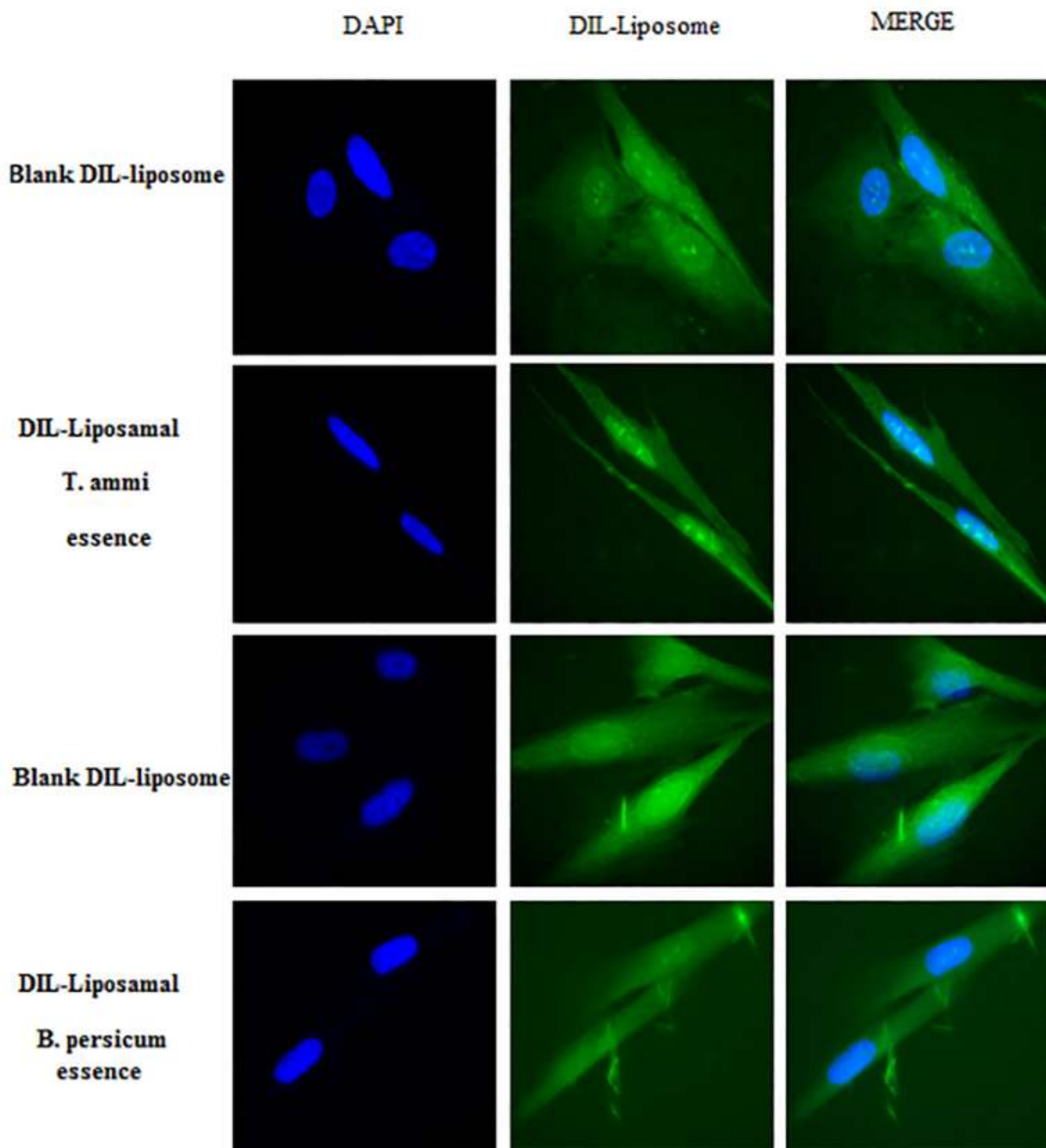


Figure 10

Fluorescent microscopic images of cellular uptake of nano-liposomes containing essential oils of *T. ammi* and *B. persicum* by human foreskin fibroblast under magnification of 400X. Cellular uptake was examined under a fluorescent microscopy, Core-shaped with DAPI color (blue) and colored DIL nanoliposomes (green) were painted. Abbreviations: DAPI, 6,4 diamidino-2-phenylindole; DIL, tetramethylindocarbocyanine perchlorate.

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