

Intensification of the antiviral activity of essential oils against parainfluenza virus PI3 using nanoencapsulation technology

Maedeh Samianifard ¹, Mohsen Lotfi ², Hasan Rafati ^{1*}

Maedeh Samianifard, Orchid ID: <https://orcid.org/0000-0001-5218-6538>

Mohsen Lotfi, Orchid ID: <https://orcid.org/0000-0001-9902-7999>

Hasan Rafati, Orchid ID: <https://orcid.org/0000-0001-6867-3963>

1-Department of Phytochemistry and Department of Pharmaceutical Engineering, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

2- Department of Quality Control, Razi Vaccine and Serum Research, Education and Extension Organization (AREEO), Karaj, Iran.

Address for correspondence: H. Rafati (ORCID 0000-0001-6867-3963), Department of Pharmaceutical Engineering, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G. C., Evin, 1983963113, Tehran, Iran

Phone: +982129904042

E-mail: h_rafati@sbu.ac.ir

Abstract:

Introduction: Enveloped viruses, including Influenza and Coronaviruses, have been considered a major source of life-threatening infections all over the world. Nanotechnology, in conjunction with natural compounds, can provide broad-spectrum virucidal and antiviral formulations to combat these potential threats. In this work, nanoemulsions prepared from 8 different essential oils (EOs) with mean particle sizes of 80-200 nm have been screened for the antiviral activities of EOs against Bovine Parainfluenza Virus type 3 (BPI3), as an enveloped virus.

Methods: Nanoemulsions, created by combining essential oils, surfactants, and water, using high energy homogenization to enhance the colloidal solubility of the essential oils, enabling a higher concentration of active ingredients. After primary screening of the antiviral activities, nanoemulsion *Thymus daenensis* Nanoemulsion (TD NE) was selected for further investigations. Quantitative PCR methods were also utilized to determine the concentration of RNA virus via the virus following TD NE formulation contacts. TEM image analysis was conducted to visualize the effect of TD NE treatment on the virus shape and probable envelop damages.

Results: After 15 minutes of treatment, two nanoemulsion formulations SK NE and TD NE, showed the lowest cytotoxicity while lowering virus count by more than 5 logarithms. The best nanoemulsion, TD NE formulation, at doses of 20 mg and 10 mg, reduced the virus titer by 4 logs over 2 and 10 minutes, respectively. The best Ct result was observed in pretreatment (36.81 ± 2.38) and then in co-treatment with the formulation (29.50 ± 2.01) compared to the negative control (40.00 ± 1.02). TEM images confirm the membrane damage to the BPI3 virus caused by TD NE formulation.

Conclusion: These findings highlight further investigations about the molecular mechanisms of interactions between active components of EOs in nanoemulsions and viral envelopes.

Keywords: Antiviral activity; Bovine parainfluenza (BPI3) Virus; Essential oil; Enveloped viruses; Nanoemulsions.

1. Introduction:

Viral diseases are known as a major cause of mortality in both humans and animals. Among different classes of viruses, enveloped viruses, including Influenza and Coronaviruses, have consistently posed a risk to human life, such recently exemplified by the COVID-19 pandemic. They pose a significant global public health emergency, followed by economic crises [1]. Bovine parainfluenza type 3 virus (BPI3) is an enveloped, single-stranded, negative-sense RNA virus belonging to the family *Paramyxoviridae* [2]. Additionally, BPI3V virion involves of a nucleocapsid surrounded by a lipid envelope with a nearly spherical shape of 150 to 200 nm, as with other parainfluenza viruses [3].

The primary method for preventing viral diseases is currently considered to be the use of live attenuated or inactivated vaccines; however, it typically does not achieve the desired effects for a prolonged period [4]. The rapid mutation rate of viruses leads to novel serotypes and genotypes, thereby reducing the effectiveness of conventional vaccines. Therefore, it is highly desirable to develop broad-spectrum multi-targeting virucidal and antiviral products to control viral outbreaks [5]. Alternatively, medicinal plants with a long history of safe use in food, pharmaceutical, and cosmeceutical industries can be considered as a safe source of new compounds for their antibacterial and antiviral properties [6].

Medicinal plants and their active ingredients are being tested in many research groups as an alternative therapy for the inhibition and treatment of infectious diseases, including viral infections [7]. Essential oils (EOs) consist of monoterpenoids and sesquiterpenoids, phenylpropanoids, flavonoids, etc. are plant-based secondary metabolites that have a variety of biological properties like antioxidant, anti-inflammatory, anti-cancer, and antibacterial effects due to their bioactive components [8]. Antiviral activity of natural compounds, including EOs like eucalyptus oil, tea tree oil, thyme oil, and some nominated monoterpenes, has been examined for herpes simplex virus (HSV) [9]. Carvacrol, a monoterpenoid phenol present in oregano oil, demonstrates virucidal properties by compromising the viral envelope and hindering replication, particularly against murine norovirus [10]. Diallyl disulfide, a sulfur-containing compound from garlic, stops viruses from getting into cells and replicating. It works against HIV and influenza by changing how the immune system works [11]. Thymol, a different monoterpenoid, works against viruses by breaking down membranes and stopping viruses from sticking to cells. This is especially true for HSV-1 and influenza viruses [12]. Menthone, a monoterpene derived from mint species, exhibits inhibitory effects on viral

enzymes and replication; however, its mechanisms are not yet fully elucidated [12]. Eugenol, a primary constituent of clove oil, exhibits significant antiviral properties by obstructing viral envelope fusion and replication, notably against HSV, hepatitis C virus (HCV), and dengue virus [13]. However, many issues need to be addressed before the successful implementation of EOs, including cytotoxicity and pro-oxidant effects, toxicity from ingestion or inhalation, volatility, hydrophobicity, and reactivity with oxygen.

Among various methods to prepare the nanoemulsions (NEs), such as homogenization, ultrasonication, and microfluidization, the nanoemulsions of EOs are generally prepared by homogenizers due to their simple processing, small particle size, and low-cost expenses. One of the magnificent reasons to use nanoemulsions is their oral bioavailability, since nanoemulsions are capable of improving the oral bioavailability of medicine with low water solubility [14]. They are fabricated as clear to opaque particles with a droplet size range of 20–500 nm in a liquid distribution system, becoming stable by an internal layer of surfactant molecules that stabilizes the droplet emulsion and inhibits phase parting [15]. Due to physicochemical stability and an additive capacity to load various active materials with long shelf life, they are a good candidate for antimicrobial or antiviral preparations [16].

Since the effectiveness of EOs of *Satureja khuzistanica*, *Thymus daenensis*, and *Mentha piperita* in nanoencapsulated form against a variety of bacteria and biofilm formation has been thoroughly assessed [17-18]. Selecting BPI3 virus involves using a membrane-enclosed virus with RNA genetic content, as well as its structural similarity to the human parainfluenza virus. Most of the herbs considered here for their antiviral activity have been previously reported in the literature or studied in our laboratory to verify their antibacterial effects [19]. Eight different EOs derived from *Satureja Khuzistanica*, *Thymus daenensis*, *Cinnamomum verum*, *Achillea millefolium*, *Mentha piperita*, *Syzygium aromaticum*, *Eucalyptus camaldulensis*, and *Allium sativum* were used in nanoemulsion formulations. Based on the studies conducted, this is the first practical report on the virucidal activity of the mentioned extracts in an in vitro experimental part against bovine parainfluenza virus type 3(BPI3).

2. Materials and methods

2.1. Materials and instruments

Satureja Khuzistanica essential oil (SKEO) was supplied by Khorraman Pharmaceutical Co., and *Allium sativum*, *Achillea millefolium*, *Cinnamomum verum*, *Eucalyptus camaldulensis*, *Mentha piperita*, *Syzygium aromaticum*, *Thymus daenensis* EOs were from Tabibdaru Kashan,

Iran. Ethylenediaminetetraacetic acid (EDTA), Dimethylsulfoxide (DMSO), Span 80, Tween 80, hydrochloric acid (HCl), and Potassium chloride (KCl), and all other chemicals were purchased from Merck Millipore (Darmstadt, Germany). Calf serum and fetal bovine serum (FBS) were purchased from Gibco E.U. Approved. An inverted microscope (Nikon TS100, Japan) was used to detect the cells and cytopathic effect. The Dynamic Light Scattering (DLS) apparatus (Nanophox Sympatec GmbH, Clausthal, Germany), Transmission Electron Microscope (TEM) (EM208S Philips, Eindhoven, The Netherlands), and high-speed homogenizer (SilentCrusher M, Heidolph, Germany) were also the other devices that were used.

2.2 GC/MS analysis:

The chemical composition of the EOs was determined using a Gas Chromatography-Mass Spectroscopy (Thermoquest-Finnigan, Manchester, UK) equipped with a fused silica capillary column (DB-5, 60 m×0.25 mm i.d.; film thickness 0.25 µm) with an ionization voltage of 70 eV and helium as carrier gas. Injector and detector temperatures were adjusted to 200 and 250 °C, respectively. The oven temperature was increased from 60 °C up to 250 °C by 4 °C/min and was held for 10 min.

2.3 Design of Experiment

Design of experiments (DOE) has become a widely used statistical approach for optimizing formulations and technical processes in research. Among the commonly applied DOE methods are orthogonal array design, Box–Behnken design (BBD), and central composite design (CCD). Based on preliminary experiments, the ranges of the independent variables were selected as follows: surfactant concentration (3.0, 5.0, and 7.0% w/w), essential oil concentration (1.0, 2.0, and 3.0% w/w), and hydrophilic–lipophilic balance (HLB) values of 8, 10, and 12. Using the BBD, 17 experimental runs were generated, including five center-point replicates to provide a reliable estimate of experimental error. Each formulation was characterized in triplicate, and the results are presented as the mean of three independent measurements. A quadratic regression model was then established to evaluate the relationship between nanoemulsion droplet size and the selected formulation variables, as described in Equation (1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

Where Y denotes the droplet size (nm); β_0 is the intercept coefficient, β_1 , β_2 , and β_3 stand for the main effect coefficients, β_{11} , β_{22} , and β_{33} are the squared coefficients, and β_{12} , β_{13} , and β_{23} show the interaction coefficients.

2.4 Preparation of NEs and stability evaluation

The preparation of nanoformulations based on the experimental design approach has already been reported in our group [19]. Different O/W nanoemulsions were prepared by mixing a surfactant mixture (Span 80+ Tween 80), different types of plant essential oils, and deionized water. The formulations are given in Table 1. Nanoemulsions were made to a total mass of 5 g, with the essential oil (2-4% w/w), surfactant (3-7% w/w), and water to 100% w/w. High-speed homogenization was performed at 10,000 rpm for 10 minutes. Briefly, a solution of deionized water with Tween 80 was added to the essential oil and Span 80 mixture to make a entire solution of 5 mL. The mixture was then homogenized for 20 minutes in a high-speed homogenizer by a 12F dispersion instrument, which was submerged into a 5 mL fluid sample at 18,000 rpm at room temperature. This method was used according to our previous study [19]. All prepared O/W formulations were characterized by dynamic light scattering (DLS). In order to evaluate the particle size, the number of nanoparticles should fit within the range of 200 to 2000 kilo count per second (Kcps), so the samples were diluted with water (1:20), and the average droplet size reported in Table 1. The obtained NEs were stored at 4 °C and monitored for particle size and physical stability for 6 months.

168 **Table 1. The composition of nanoemulsion formulations (F1-F9) and their droplet size after preparation.**

Number	Essential oil	Surfactant (%W/W) Span 80+Tween 80	Oil (%W/W)	Water (%W/W)	Size (nm)	PDI
F1	<i>Satureja Khuzistanica</i>	3.0	2.0	95.0	116±3.5	0.27
F2	<i>Allium sativum</i>	4.6	3.0	92.4	78±2.4	0.28
F3	<i>Thymus daenensis</i>	3.0	2.0	95.0	134±3.8	0.23
F4	<i>Cinnamomum verum</i>	5.0	2.0	93.0	161±4.4	0.16
F5	<i>Eucalyptus camaldulensis</i>	4.6	3.0	92.4	101±2.9	0.16
F6	<i>Achillea millefolium</i>	4.6	3.0	92.4	78 ±3.2	0.20
F7	<i>Mentha piperita</i>	7.0	4.0	89.0	104±3.3	0.19
F8	<i>Syzygium aromaticum</i>	4.6	3.0	92.4	92±4.3	0.23
F9	-	5.0	-	95.0	-	-

169 2.5 Cell culture and BPI3 virus propagation

170 The Razi bovine kidney (RBK) cell line (NCBI code: C541) was cultured in Dulbecco's
 171 Modified Eagle's Medium (DMEM) supplemented with 10% (w/w) calf serum, 100 IU/mL
 172 penicillin, and 100 µg/mL streptomycin. Cells were maintained in a humidified incubator at 37
 173 °C with 5% CO₂ and were routinely passaged two to three times per week to maintain optimal
 174 growth.

175 Bovine parainfluenza virus type 3 (BPI3) was prepared at Razi Vaccine Institute, Alborz, Iran.
 176 BPI3 is one of the major viral agents causing the bovine respiratory disease complex. The
 177 sample was propagated in RBK cells with MOI 0.01 in DMEM cell culture supplemented with
 178 2 %w/w calf serum, 100 IU/mL penicillin, and 100 µg/mL Streptomycin in a humidified
 179 incubator with 5 % CO₂ at 37 °C. After one week, the culture medium was centrifuged at 3000
 180 rpm for 15 minutes to remove cell debris. Then, the supernatant containing BPI3 viral particles
 181 was separated and aliquoted to be stored as an active virus stock solution at -70 °C for use in
 182 investigating the effect of nanoemulsions on BPI3 virus, whose titer was 10⁷ CCID₅₀/mL (50

% cell culture infectious dose). This sample was also used as a positive control in the work steps.

2.6 Cytotoxicity assay of nanoemulsions on RBK cells

The MTT assay is a colorimetric measurement used to assess the metabolic activity of a cell. The activity of NAD (P) H-dependent oxidoreductase enzymes is used to evaluate cell viability [20]. The cytotoxicity of the nanoemulsion formulations (F1-F8) and the control group (F9) was tested against the RBK cells using the MTT test. In a 75 mL flask RBK cells were cultured at 37 °C and 5 % CO₂ for 48 h in a humidified environment. The cells were proliferated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 IU/mL penicillin, 100 µg/mL Streptomycin and 10 %v/v fetal calf serum (FBS).

The cells were counted at a density of 2.5×10^6 cells /mL of DMEM, which was included with 10 %v/v fetal calf serum (FBS) and 1 %w/v Penicillin/ Streptomycin. 100 µL of the previous cell medium was added to each well of a 96-well microplate and incubated at 37 °C with 5 % CO₂ for 48 h. Then, the cell attachment and morphology were checked. The dilutions (1/2 to 1/4096) of each nanoemulsion and control were added to the cells in a basic culture medium, after removing the media. At the same time, DMEM was used as the negative control. Concentrations are reported as mg of total nanoemulsion formulation per mL. All plates were incubated at 37 °C with 5% CO₂ for 48 h. After desired time, the medium of the wells was removed, and 40 µL PBS included MTT (5 mg/mL) was added to all wells. 150 µL of DMSO was added to each well after the plates were incubated at 37 °C with 5 % CO₂ for 4 h. The plates were located for 10 min on shaker incubator, and the absorbance at 570 nm was determined using a microplate reader. Cell growth inhibition for each formulation was defined as the CC₅₀/mL value, which is defined as the concentration that reduces cell viability by 50%.

2.7 Antiviral activity value determination

The assay technique was based on standard techniques for the evaluation of chemicals for antiviral activity [21]. In this method, 0.5 mL of BPI3 virus with a titer of 10^7 CCID₅₀ /mL was added to 2 mL of various dilutions of every nanoemulsion formulation. The mixtures were then incubated for 15, 30, and 60 minutes at room temperature. After the required time had elapsed, the next step involved adding 0.5 mL of this solution to 4.5 mL of the DMEM solution that was previously kept at 4 °C. Subsequently, dilutions ranging from 10^{-1} to 10^{-7} were made from this solution. 100 µL of each dilution was transferred into a 96-well plate in four replicates. After preparing all the dilutions, 100 µL of RBK cells were added to each well, resulting in a

total of 30000 cells/well. After incubating the microplate in a CO₂ incubator at 37 °C with suitable humidity for 5 to 7 days, the presence of active viruses was detected through the observation, recording, and calculation of cytopathic effects using the Reed and Muench method [22]. CCID₅₀ /mL, which was calculated based on this method ($P < 0.01$). Concentrations are reported as mg of total nanoemulsion formulation per mL.

A positive control solution was prepared using a virus and medium. Obtained values were normalized with the control expressed in the decrease of CCID₅₀ /mL. The cells without formulations and virus exposure (only media) served as the negative control.

2.8 Real-time PCR

RBK cells were cultured in five 25 mL flasks with DMEM media and 10 % calf serum, placed in a CO₂ incubator at 37 °C, which had approximately 3.5×10^6 cells in total after 24 h. The first flask was used as the positive control of the effect of the virus, without the presence of nanoemulsion on the cell. The second one was used for cell control. Three of the cell flasks were used to examine the interaction effects between the virus and the nanoemulsion formulation F3 (*T. daenensis*) solution in 3 different conditions: pretreatment, cotreatment, and posttreatment. 200 µL of each virus suspension was added to the cell monolayer and incubated at 37 °C for 1 h. After an hour, the supernatant solution was washed off, and the cells were treated with culture medium and serum. The cells were then placed in a CO₂ incubator at 37 °C for 5 to 7 days, and the cytopathic effects were daily investigated. After this step, 200 µL was removed from the supernatant solution, and the subsequent steps were performed in the order specified by the RNA extraction kit. Viral RNA extraction was done with the DynaBio Kit (Viral DNA/ RNA Extraction Mini Kit, Iran) as instructed in the company's guidelines. Briefly, 200 µL of cell culture supernatant was vortexed with Binding Buffer (BB) that contained poly-A and proteinase K and then incubated for 10 min at 56 °C. The lysate mixture was moved to a column placed in a 2 mL collection tube and centrifuged at 6000 x g for 1 min (2 times). The column was centrifuged after adding an inhibitor removal buffer (IRB). The column was washed and centrifuged twice at 13,000 x g for 1 min to dry the column matrix. At the end, the column was eluted by adding elution buffer and centrifuged for 1 min at 8000 x g. The eluted RNA was aliquoted and stored at -80 °C until further analysis. Real-time reverse transcription-PCR was applied using 100 ng of RNA and the Two-step qPCR kit. Fovorgen kit (England) was used for cDNA synthesis. The real-time PCR reactions were

achieved using the following primers and probes, fluorophores, quenchers and sequences used are shown in Table 2 for the study of the viral strain BPI3 [23].

Table 2. Primers/probes for real-time PCR

Primers/probe	Sequences (5'-3')
BPI3 Forward2	TGATTGGATGTTTCGGGAGTGA
BPI3Reverse2	AGAATCCTTTCCTCAATCCTGATATACT
BPI3Fam	FAM-TACAATCGAGGATCTTGTTCa-MGBNFQ

Real-time PCR cycling conditions were followed using these steps: 95 °C for 5 min, 95 °C for 15 s, and 40 cycles of 60 °C for 60 s. Real-time PCR was performed by the ABI real-time PCR 7500 sequence detection system, and the data were studied by the ABI 7500 SDS software (Applied Biosystems).

2.9 Transmission electron microscopy (TEM)

By TEM method the morphology of the best nanoemulsion formulation (F3), as well as the pure BPI3 virus, and following 60 minutes of pretreatment with the most effective nanoemulsion formulation F3, were evaluated. One drop of each virus sample was placed on a copper grid and then stained with a 2% formaldehyde solution for 1 minute. At an acceleration voltage of 100 kV, the grid was detected. Transmission electron microscopy (TEM) was conducted using a Philips EM208 S electron microscope (Philips, Eindhoven, Netherlands).

2.10 Statistical analysis

Each experiment was performed in quadruplicate and repeated three times. Data are reported as mean $\pm$ SD by Microsoft Excel. The GraphPad Prism 9 software calculated CC₅₀ values. Statistically analyzed by one-way ANOVA, sample values were considered significant with $P < 0.05$. Design-Expert Software (State-Ease Inc., version 7.0.0) was used to examine the experimental data and estimate the predicted responses.

3. Results

3.1. Gas chromatography-mass spectroscopy GC/MS analysis of EOs

GC-MS, as a sensitive and specific analytical technique, was used to explore the chemical profile of the EOs. The major components of the EOs were determined to be (Area percent); *Satureja Khuzistanica* (Carvacrol; 87.65%), *Allium sativum* (Diallyl di, tri, and tetra sulfide; 56.47%), *Thymus daenensis* (Thymol; 53.20%, Para-cymene; 25.20%), *Cinnamomum verum* (Cinnamaldehyde; 70.75%), *Eucalyptus camaldulensis* (1,8- cineol; 54.65%), *Achillea millefolium* (Camphor; 22.68%, 1,8- cineole; 25.36%), *Mentha piperita* (Menthone; 38.37%, Menthol; 37.08%), *Syzygium aromaticum* (Eugenol; 72.58%). Table 3 summarizes the main chemical composition of the EOs.

Table 3. Main chemical composition of the Essential oils using GC/MS analysis

No.	Compounds (Area %)
F1	Carvacrol (87.65), P-cymene (6.50), linalool (2.70).
F2	Diallyl disulfide (15.91), Diallyl trisulphide (24.71), Diallyl tetrasulfide (15.85), (+)- δ -Cadinene (5.65).
F3	Thymol (53.20), P-cymene (25.35), Carvacrol (3.83).
F4	Cinnamaldehyde (70.72), Cinnamyl acetate (5.7), β -caryophyllene (5.4), Linalool (2.8).
F5	1,8-cineol (54.65), α -pinene (10.15), Limonene (5.17), β -pinene (3.30), p-cymene (2.1), α -terpineol (1.80)
F6	Camphor (22.68), 1,8-cineol (17.90), α -pinene (14.34), p-cymene (6.30), Subinene (6.23), Borneol (5.28), Chamazulene (4.69), Terpinene-4-ol (5.78).
F7	Menthone (38.37), Menthol (42.10), Isomenthone (6.80), p-cymene (2.10).
F8	Eugenol (72.58), Eugenol acetate (8.54), β -Caryophyllene (7.23).

3.2. Nanoemulsion characterization

A double-beam dynamic light scattering (DLS) system was used to estimate the particle size and size distribution of the nanodroplets. The samples were diluted to achieve droplet counts ranging from 200 to 2000 kCPS (Figure 1). Fresh-made nanoemulsion formulations were used to evaluate the biological activity throughout the studies. TEM image of F3 formulation (Figure 2) presents spherical and monodisperse nanodroplets of *Thymus daenensis* EO in the sample. Morphology, size and size distribution of the nanoemulsion droplets established by TEM image and using image analysis shows slightly smaller particle size than the results reported using DLS analysis (Table 1, Figure 1), which can be explained by the fact that DLS technique evaluates the hydrodynamic particle size of the nanoparticles compared to the real TEM images of the particles.

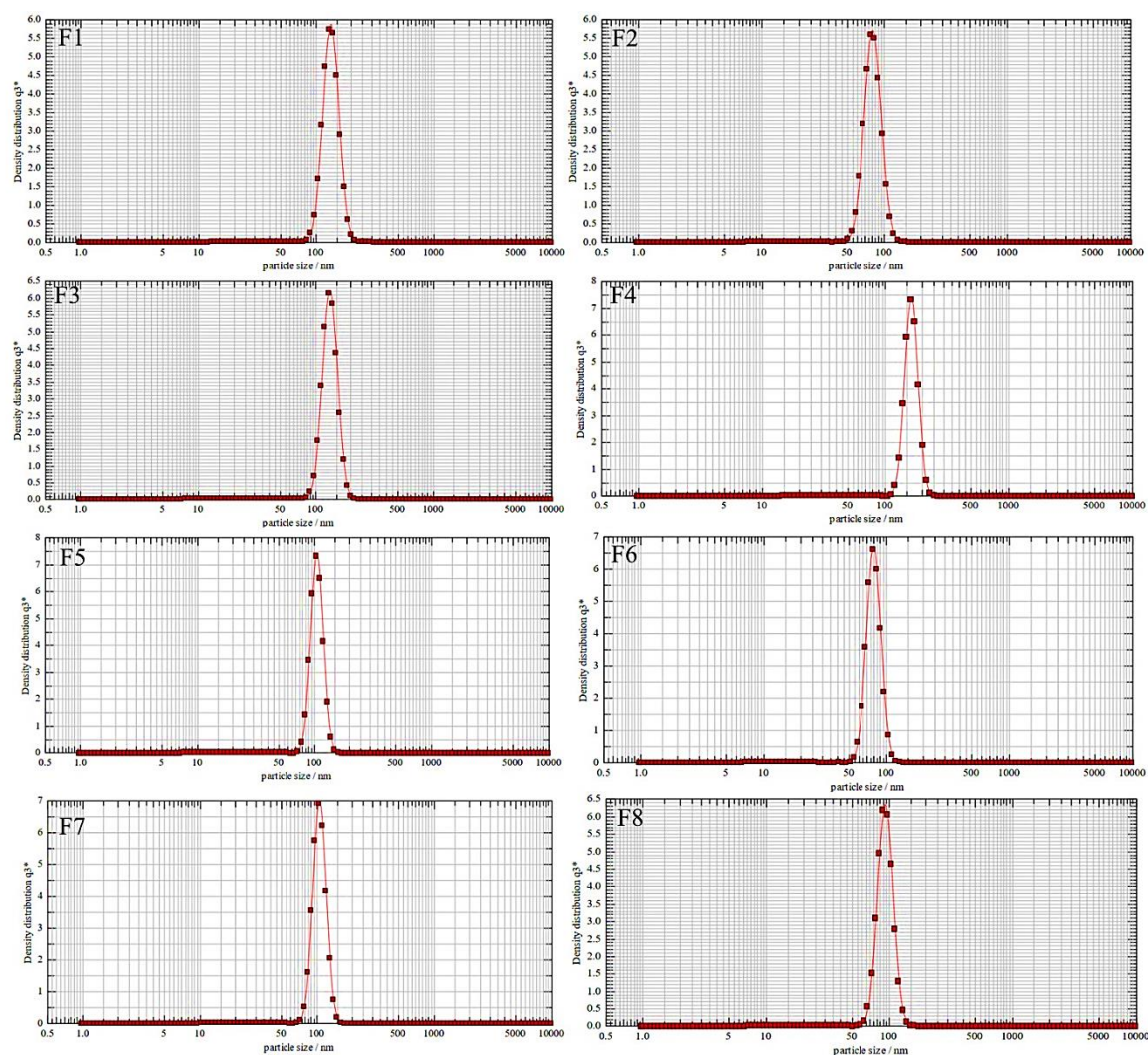


Figure 1- The mean droplet size of the determined using DLS were (F1) 129 ± 3.5 nm, (F2) 78 ± 2.4 nm, (F3) 134 ± 3.8 nm, (F4) 161 ± 4.4 nm, (F5) 101 ± 2.9 nm, (F6) 78 ± 2.4 nm, (F7) 104 ± 3.3 nm and (F8) 92 ± 4.3 nm.

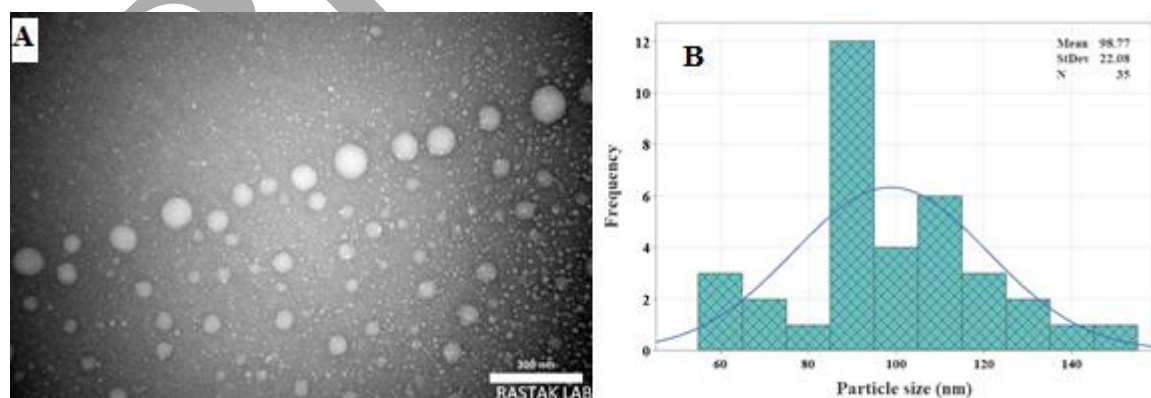


Figure 2- TEM of *Thymus daenensis* EO Nanoemulsions (A) and Size distribution (B) analysis.

3.3. Optimization of the most biologically active nanoemulsion formulation (*T. daenensis* EO nanoemulsion)

The Box–Behnken design (BBD), a response surface methodology (RSM)-based experimental design, was employed to optimize the nanoemulsion formulation by identifying the droplet size that provided the greatest biological activity and physicochemical stability. For the combination of the independent factors, i.e., the percentages of surfactant (A), essential oil (B), and (C) HLB value given in Table 4, the droplet size of the nanoemulsion as a single response was determined by the DLS technique. All measurements were performed in triplicate, and the average droplet size was reported.

Table 4. Box–Behnken design plan, experimental and predicted results

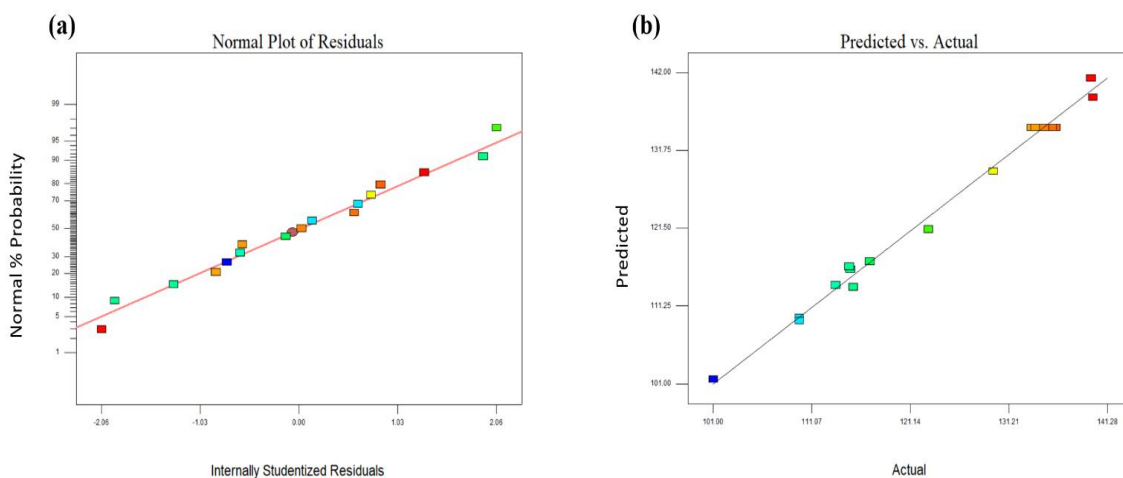
Run	Factors			Response	
	Surfactant (w/w %) A	Essential oil (w/w %) B	HLB C	Droplet size (nm)	Predicted
1	5.00	3.00	8	139.6	138.2
2	5.00	2.00	10	133.5	133.70
3	3.00	2.00	8	129.6	128.7
4	3.00	1.00	10	117	117.6
5	7.00	3.00	10	109.8	109.04
6	7.00	2.00	8	115.3	115.9
7	5.00	2.00	10	135.6	136.7
8	5.00	2.00	10	136	136.37
9	3.00	3.00	10	139.8	139.5
10	7.00	1.00	10	104.30	104.89
11	7.00	2.00	12	113.80	113.90
12	5.00	1.00	8	115	115.52
13	5.00	2.00	10	135.9	135.35
14	5.00	1.00	12	113.50	113.56
15	5.00	3.00	12	134.8	134.31
16	5.00	2.00	10	133.9	133.7
17	3.00	2.00	12	114.9	114.5

326 The experimental design consisted of 17 runs, including five center-point replicates, as
 327 presented in Table 4. The relationships between the independent variables and the
 328 nanoemulsion droplet size were described using second-order polynomial models. Equations 4
 329 and 5 present these models in terms of both actual and coded factor values.

$$\begin{aligned} \text{Droplet size of nanoemulsion (Actual)} = \text{Mean droplet size} = & -266.95688 \\ & +28.78125*\text{Surfactant} +86.60750*\text{Essential oil} +52.83125*\text{HLB} -3.50000* \\ & \text{Surfactant}*\text{Essential oil} +0.025000*\text{Surfactant}*\text{HLB} -4.91250*\text{Essential oil}*\text{HLB} \\ & -2.57937*\text{Surfactant}^2 -4.04250*\text{Essential oil}^2 -2.31063*\text{HLB}^2 \end{aligned} \quad (4)$$

$$\begin{aligned} \text{Droplet size of nanoemulsion (Coded)} = \text{Mean droplet size} = & +134.76 -7.53A + \\ & 3.81B -6.16C -7.00AB +0.100AC -9.82BC -10.32A^2 -4.04B^2 -9.24C^2 \end{aligned} \quad (5)$$

330 The typical likelihood plot, which determines whether the residuals are part of a normal
 331 distribution, requires that the data points be in a straight line (Figure 3a). A plot of the expected
 332 versus actual results is shown in (Figure 3b), which helps identify a value or set of data that the
 333 regression model cannot accurately forecast. These plots also demonstrate that there is an
 334 excellent match between the expected values and the experimental data.



335 Figure 3- Normal plots (a) and Pred vs. Actual graphs (b) for droplet size response.

Table 5 presents the ANOVA results for the quadratic response surface model. The significance of the model was assessed using the p-value and F-value. The model was found to be highly significant, as indicated by its very low p-value ($p < 0.0001$) and high F-value (101.16). In addition, the lack-of-fit test was not significant ($p = 0.1035$) relative to the pure error, suggesting that the model adequately describes the experimental data and that no systematic deviation exists between the observed and predicted responses.

The coefficient of determination (R^2) was used to evaluate the model's goodness of fit. An R^2 value of 0.9924 indicates an excellent agreement between the experimental results and the predicted values, with more than 99% of the variability in the response explained by the model. Other statistical parameters further support the model's reliability, including a standard deviation of 1.63, a coefficient of variation (CV) of 1.32%, an adjusted R^2 of 0.9826, a predicted R^2 of 0.9050, and an adequate precision value of 31.725. Together, these results demonstrate that the model possesses high accuracy, good predictive capability, and acceptable precision. Overall, the statistical findings confirm that the developed quadratic model is robust and reliable. It provides a strong basis for interpreting the response surface plots and can be confidently used to predict the optimum processing conditions required to maximize the stability response.

Table 5. ANOVA results for independent variables obtained from RSM.

	Sum of Squares	df	Mean Square	F Value	p-value, Prob > F	
Model	2419.202853	9	268.800317	101.1641	< 0.0001	significant
A-Surfactant	453.005	1	453.005	170.4903	< 0.0001	
B-Essential oil	116.28125	1	116.28125	43.76294	0.0003	
C-HLB	303.81125	1	303.81125	114.3406	< 0.0001	
AB	196	1	196	73.76542	< 0.0001	
AC	0.04	1	0.04	0.015054	0.9058	
BC	386.1225	1	386.1225	145.3188	< 0.0001	
A²	448.2139211	1	448.2139211	168.6872	< 0.0001	
B²	68.80760526	1	68.80760526	25.89603	0.0014	
C²	359.6791842	1	359.6791842	135.3668	< 0.0001	
Residual	18.5995	7	2.657071429			
Lack of Fit	14.0275	3	4.675833333	4.090843	0.1035	not significant
Pure Error	4.572	4	1.143			
Cor Total	2437.802353	16				

Figure 4a shows the effect of essential oil and surfactant percentage on nanoemulsion particle size. In low essential oil concentrations, droplet size was initially increased and then decreased by increasing surfactant concentrations. Because the surfactant promotes the emulsification process, the initial rise in droplet size with increasing surfactant concentrations may occur in low essential oil concentrations. The development of larger initial droplets may result from an increase in surfactant content. However, when more surfactant improves the stability and dispersion of the droplets in the mixture.

This effect on droplet size, however, had a constant reduction effect with increasing oil concentrations. The capacity of the surfactant to lower the surface tension between the oil and the water phase is responsible for the droplet size decrease that occurs with increasing surfactant concentrations in high essential oil concentrations. Finer oil droplets are produced as a result of improved oil droplet dispersion brought about by this decrease in surface tension. Potentially because the formulation may be dominated by the oil phase, producing bigger oil

droplets, increasing the concentration of essential oil in a formulation with minimal surfactants increase in particle size. The emulsion's propensity to fracture due to a lack of surfactants to stabilize and decrease the droplet size may also result in greater particle sizes as smaller droplets combine to form larger ones.

Figure 4b shows the Influence of Hydrophilic-Lipophilic Balance (HLB) and surfactant percentage on nanoemulsion particle size. The hydrophilicity of surfactants is generally increased by raising their HLB value, which encourages the creation of smaller particles during the emulsification or dispersion processes. This is because a narrower size distribution results from stronger HLB surfactants stabilizing the dispersion better and avoiding particle coalescence. Because greater HLB surfactants improve the stability of smaller droplets and more efficiently lower interfacial tension, increasing surfactant mainly reduces particle size in systems with higher HLB values. Because lower HLB surfactants are more lipophilic and less successful at stabilizing smaller particles in aqueous settings, increasing surfactants at lower HLB values might not have a substantial impact on particle size; however, a decrease in particle size in these conditions is also demonstrated.

Figure 4c shows the effect of HLB and essential oil percentage on nanoemulsion particle size. Because EOs have a propensity to agglomerate, increasing their content in formulations with low HLB values may result in bigger particle sizes. Oil droplets are more prone to agglomerate as their concentration rises because low HLB surfactants are less effective in stabilizing oil-in-water emulsions. As the surfactant's capacity to stabilize the emulsion declines, this may lead to bigger particle sizes. Because EOs have better emulsifying qualities, increasing their concentration might result in a decrease in particle size in systems with high HLB values. Smaller oil droplets are stabilized by high HLB surfactants, which encourage homogeneous dispersion and inhibit coalescence. As a result of a higher affinity for water, which enables more efficient emulsification of the hydrophobic essential oils, increased HLB values tend to

reduce particle size in high essential oil concentrations. This improved emulsification makes it easier for smaller droplets to form. However, the effects would not be as noticeable at low concentrations of essential oil because the emulsifying agents would not be able to completely overcome the oil phase's insufficient volume to interact effectively, which would result in less noticeable variations in droplet size with HLB variation.

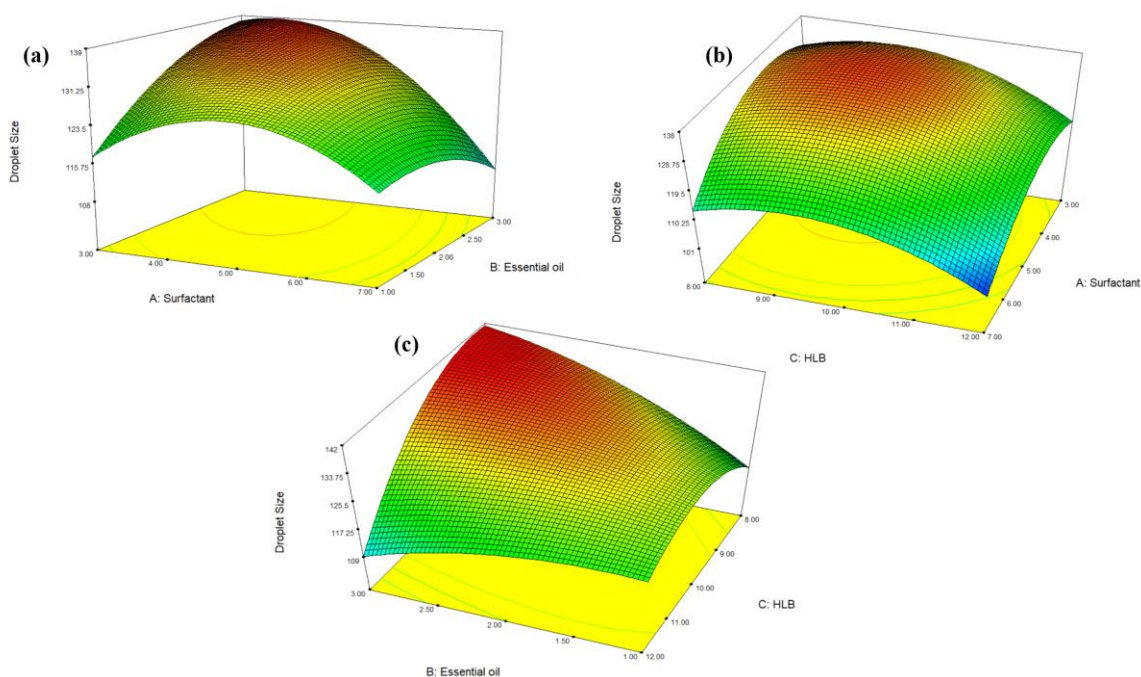


Figure 4. 3D plots and interaction effect of (a) surfactant and essential oil, (b) surfactant and HLB, (c) essential oil and HLB on the droplet size

3.4. Evaluation of the cytotoxicity of F1-F9 on RBK cells

To assess the effect of the nanoemulsions on the viruses using different treatment conditions, primarily the cytotoxicity of the nanoemulsion formulations on the host cells should be evaluated. RBK cells were used to conduct the MTT test to evaluate the cytotoxicity of the produced nanoemulsions. Based on data from Table 6, the MTT test was used to examine the impact of the nanoformulations with varying essential oil concentrations on the viability of the cell culture. Table 6 presents the CC_{50} mg/mL values for the formulations versus the cytotoxicity of the mixture of Span 80 and Tween 80 (F9) as the control group to measure the toxicity of surfactants without the presence of essential oils. The results show that the control group ($CC_{50}=3.12\pm0.087$ mg/mL) possesses the least cytotoxicity compared to some of the formulations. The outcomes were signified as mean $\pm$ SD, and $p < 0.05$.

Table 6. Measurements of CC₅₀ (50% cytotoxicity concentration) Doses represent mg of total formulation per mL.

Formulation number	Essential oil	CC ₅₀ (mg/mL)
F1	<i>Satureja Khuzistanica</i>	0.304±0.057
F2	<i>Allium sativum</i>	0.149±1.531
F3	<i>Thymus daenensis</i>	0.613±0.083
F4	<i>Cinnamomum verum</i>	0.166±0.031
F5	<i>Eucalyptus camaldulensis</i>	0.165±1.23
F6	<i>Achillea millefolium</i>	0.279±0.075
F7	<i>Mentha piperita</i>	0.232±0.026
F8	<i>Syzygium aromaticum</i>	0.189±0.047

3.5. Effect of nanoemulsions on BPI3 virus as an antiviral agent

Screening and selecting the procedures were performed in different dilutions of nanoemulsions, and the time of interaction between the virus suspension and the formulation. The decrease in virus infectivity was designed by subtracting the logarithmic virus titers before and after the treatment. Figure 5 shows the decrease in Log CCID₅₀ /mL titration results of formulations F1-F8 and F9 as a surfactant control group on the BPI3 virus at 15, 30, and 60 min in a pretreatment situation. BPI3 virus, positive control titer was 10⁷ CCID₅₀/mL. Figure 5 illustrates the decrease in the virus titer caused by nanoemulsion compared to the virus control. As can be seen in Figure 5, the F3 formulations containing *T. daenensis* essential oil showed the most neutralization capacity in the shortest time. After 60 minutes of pretreatment, the interaction between the BPI3 virus and the formulations, only formulations F1 and F3, could neutralize and reduce the titer by 4 logs. These formulations may be considered as potential virucidal formulations with the least cytotoxic activities.

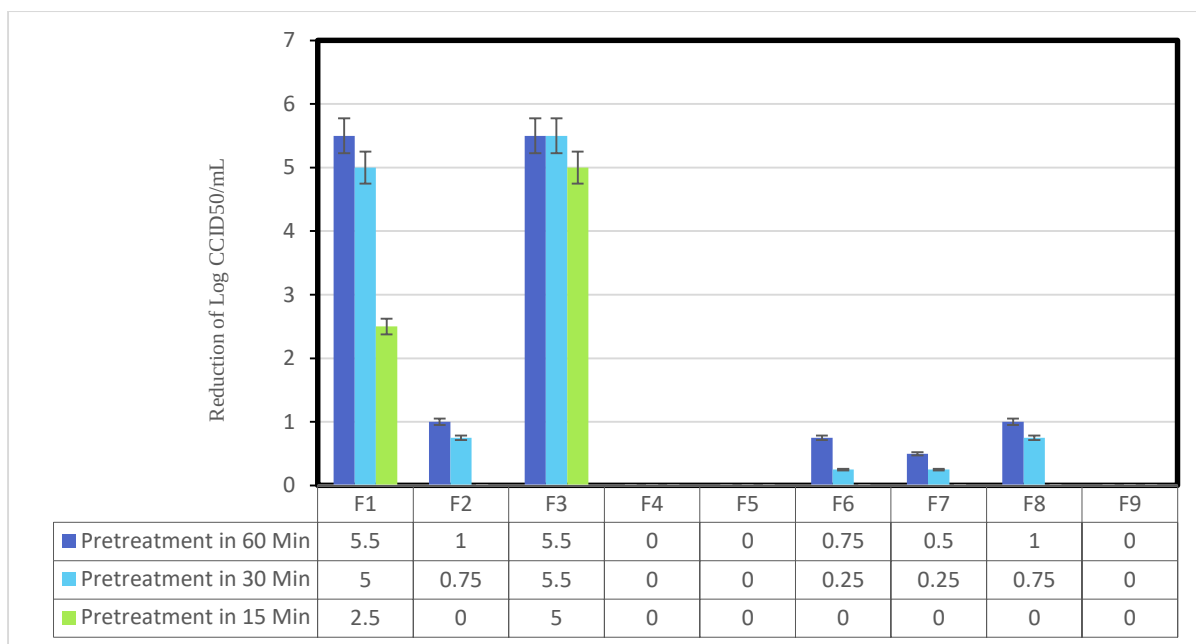


Figure 5. Reduction of Log CCID₅₀ mL⁻¹ in vitro antiviral assay results for 9 nanoemulsion formulations against BPI3 virus in pretreatment situations in 15, 30, and 60 min. BPI3 virus (positive control) titer was 10⁷ CCID₅₀/ml.

Since the F3 formulation was the most effective preparation with the least cytotoxicity, it was studied to determine the lowest optimal dose that could achieve high effectiveness in a shorter time. Figure 6 shows the effect of the F3 formulation at concentrations of 20 and 10 mg/mL over time, demonstrating the nanoemulsion F3's ability to reduce 4 logs in 2 and 10 minutes, respectively. These results reveal the concentration-dependent inhibitory effects of the F3 formulation.

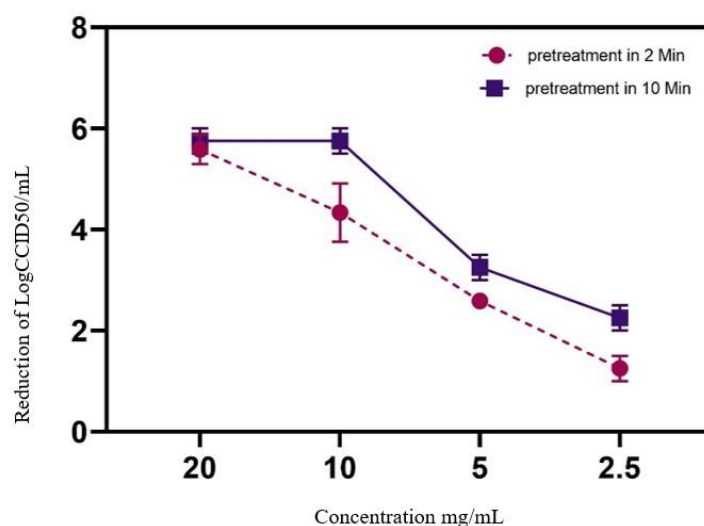


Figure 6. The reduction of LogCCID₅₀/mL of F3 (*Thymus daenensis* formulation) for a series of dilutions: 1/5 (20 mg/mL), 1/10 (10 mg/mL), 1/20 (5 mg/mL), and 1/40 (2.5 mg/mL).

3.6. Quantitative PCR analysis

The antiviral efficacy of the essential oil nanoemulsion (F3) was assessed using quantitative PCR, which revealed a significant reduction in viral RNA content upon interaction with the nanoemulsion, as shown in Table 7. The outcome motivated consideration of the antiviral activity of *T. daenensis* formulation (F3) at the molecular level in pre-, co-, and post-treatment with the BPI3 virus. The results of this study indicated significant differences in three states of the interaction between the BPI3 virus and F3 formulation, which is more effective in pretreatment and cotreatment compared to post-treatment. Cycle threshold (CT) values for the BPI3 virus were analyzed under three interaction conditions using the F3 nanoemulsion obtained from *T. daeensis*, in addition to positive and negative controls. In the positive control (virus only), the CT value was 16.53 ± 2.58 , indicating an increase in viral RNA levels. The negative control (cells only) recorded a CT of 40.00 ± 1.02 , confirming the absence of virus detection. Pretreatment resulted in almost complete inhibition of viral RNA, indicating the absence of infection. Co-treatment resulted in a significant reduction in the viral replication, although it did not completely eradicate it. This reduction in viral RNA content suggests that the selected nanoemulsion can possibly disrupt the viral structure and have effect on viral replication, ultimately leading to a decrease in the viral load. Post-treatment showed a negligible suppression, indicating that the F3 formulation is more effective in preventing infection at early stages than in reducing established viral loads. Formulation F3 shows significant potential for preventive use and early intervention, while its effectiveness decreases after the onset of the viral infection.

Table 7. Cycle thresholds in 3 different states of interaction between BPI3 virus and F3 formulation (*T. daenensis* nanoemulsions)

Treatment	Ct value (mean $\pm$ SD, $p < 0.01$)
BPI3 virus (positive control)	16.53 $\pm$ 2.58
Cell (negative control)	40.00 $\pm$ 1.02
Pre-treatment	36.81 $\pm$ 2.38
Co-treatment	29.50 $\pm$ 2.01
Post-treatment	19.35 $\pm$ 1.98

3.7. Transmission electron microscopy

Transmission electron microscopy was utilized to assess the impact of *T. daenensis* formulation (F3) on the BPI3 virus to investigate the possible disruption of enveloped virus membranes (Figure 7). TEM characterization of BPI3 pure virus showed the spherical and homogeneous shape of particles, Figure 7a. On the other hand, Figure 7b revealed the effect of *T. daenensis* nanoemulsions after 1-hour pretreatment on BPI3. The images show that the BPI3 virus membrane is damaged following the nanoemulsion treatment.

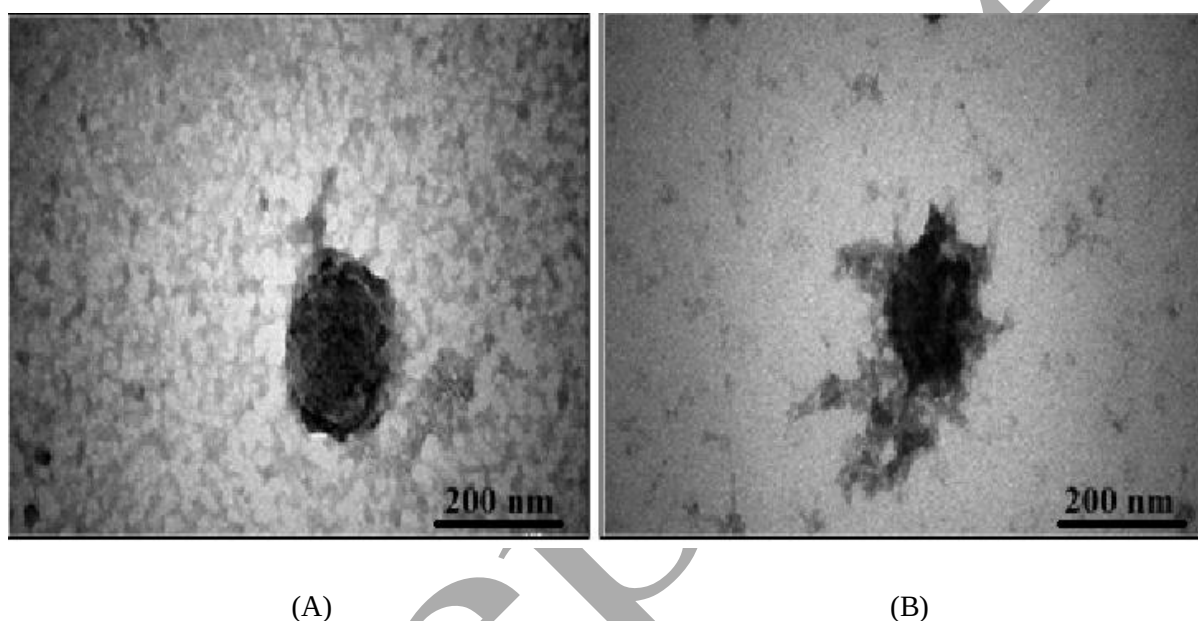


Figure 7. The TEM image of pure BPI3 virus (A) and pretreated pure BPI3 virus for an hour (B).

4. Discussion

Nanoemulsions have gained significant attention for their potential to combat various microorganisms, including viruses. The nanoemulsions of plants' EOs can play a significant role in improving the dispersibility of these hydrophobic compounds in aqueous solutions, which is the main biological medium. Two immiscible fluids are blended to create a single phase by adding an emulsifying agent, i.e., surfactant and co-surfactant [24]. In this study for the nanoemulsions, numerical optimization to find out the most stable and biologically active nanoemulsion composition, the droplet size was considered maximized in line with minimized surfactant percentage, maximized HLB, and an average amount of nanoemulsions.

Moreover, the viability results in this study confirmed that the survival rate was observed at higher concentrations when utilizing essential oil in nanoemulsions compared to the pure EO, as previously reported [25]. The mortality rate is rising in tandem with an increase in the

percentage of EOs. Although *S. khuzistanica* essential oil and its main components have the potential for further usage in antibacterial applications, the toxicity in normal cells is noteworthy [26]. Furthermore, the nanoemulsions exhibited limited cytotoxicity on the tested cell line, suggesting their safety for potential therapeutic usage. It is crucial to emphasize that transitioning from in vitro results to in vivo conditions requires addressing additional concerns, including bioavailability, metabolism, half-life, and potential systemic toxicity of a formulation within a complex living organism.

We have previously shown the effect of *T. daenensis* nanoemulsion formulation on a Gram-negative bacterium, *E. coli*, [27] and then compared the antibacterial activities on a Gram-positive, *Bacillus atrophaeus*, and a Gram-negative, *E. coli* bacterium. It was proposed that the same nanoemulsion had antibacterial activity through interaction with the bacterial cell membrane, which was approved by the results of DESI analysis of the lipid membrane [28]. This led us to assume that the nanoformulations of EOs may also affect the lipid bilayer of the enveloped viruses. However, the results showed that in addition to the physicochemical properties and particle size of the nanoemulsions, the nature of the essential oil is also of particular importance. *T. daenensis*, a member of the Thymus genus, is frequently encountered in Iran and is valued for its medicinal and aromatic qualities [29]. In traditional Persian medicine, *T. daenensis* is also used as a herbal tea, food component, and medicinal plant for its reputed therapeutic properties [30]. Ultimately, a product is considered to be antiviral and/or virucidal when the treatment with the product leads to an equivalent or more than 4 log reductions in the virus titer [21].

The balance between virus stability and RNA release is essential for a virus's proliferation cycle. Therefore, agents that disrupt the lipid structure might be considered antiviral agents [31]. Interestingly, a comparative study of the antiviral effects of 15 plant extracts on avian infectious bronchitis virus (IBV) with the same RT-PCR technique showed that this technique is significantly efficient for antiviral study purposes [7]. Compared to bacterial infections, viral infections are more difficult to treat. Compounds derived from natural sources have gained attention for their potential to manage viral infections in modern society as alternatives to synthetic medications. In this way, the finding may lead to a novel plant-based nanoemulsion that inactivates the tested enveloped virus BPI3.

These data indicate that the F3 formulation probably disrupts the virus surface structure and virion morphology. Our research studies demonstrating the effect of nanoemulsions on the

membranes of Gram-negative and Gram-positive bacteria by DESI provided direct mechanistic evidence that confirmed the disruption of lipid bilayers in Gram-negative bacteria [28]. Based on this approach, it seems that the phospholipid membrane of the virus is probably affected by the collision of nanoemulsion particles and the action of the active ingredients, although more experiments are needed to accurately validate this hypothesis. Enveloped viruses are typically easier to inactivate due to the lipid envelope being the most susceptible part of the virus particle.

5. Conclusion

This study highlights the potential of essential oil nanoemulsions as a virucidal agent against the BPI3 virus. It has also been shown that among 8 different essential oils, the conversion of *T. daenensis* essential oil into a nanoemulsion greatly enhanced its virucidal activity among the prepared formulations. Moreover, *T. daenensis* nanoemulsion can inhibit BPI3 as an enveloped virus, perhaps by disrupting viral envelope lipid bilayers. It has been postulated that the nanoparticles with the minimum particle size provide better interaction with the viruses' envelopes. This may be because steric hindrance provided by high concentrations of surfactants may prohibit the optimum interaction of active components with the virus's membrane. From the results, it can be concluded that nanoencapsulation can enhance the antiviral activity of EOs when compared with the non-encapsulated forms, through several mechanisms. Nanoencapsulation enhances the aqueous solubility and stability (less volatility and phase separation), improves availability and interactions with the viral and cellular targets, and provides a potential synergism from the direct physical disruption of the viral membranes by nanoscale droplets.

Despite the potential of nanoemulsions as a virucidal preparation, further investigations into the molecular mechanism of the nanoemulsions' active components and the lipid bilayer of the enveloped viruses are warranted.

Acknowledgements

The authors would like to thank the colleagues of the Razi Vaccine and Serum Research Institute, Karaj, Iran, for their assistance in advancing this research.

Authors' Contribution

M.S.: study conception and design, data collection, analysis, and interpretation of results, and manuscript preparation. M. L.: Virus resources, Validation, Formal analysis, Visualization. H.

572 R.: Conceptualization, NE resources, Supervision of the study, reviewing and editing the
573 manuscript.

574 **Conflict of interest**

575 The authors declared no conflict of interest.

576 **Ethics approval**

577 This study did not involve human participants or live animals. All procedures were performed
578 using established in vitro cell culture techniques. Therefore, ethics approval was not required.

579 **Grant support or Funding/Support**

580 No funding has been received.

581 **Data availability statement**

582 Data of the present study will be available upon request from the corresponding author.

583

584

References:

- [1] Lu L, Su S, Yang H, Jiang S. Antivirals with common targets against highly pathogenic viruses. *Cell*. 2021; 184(6):1604-1620. [DOI:10.1016/j.cell.2021.02.009] [PMID]
- [2] Rima B, Collins P, Easton A, Fouchier R, Kurath G, Lamb RA, et al. ICTV virus taxonomy profile: Pneumoviridae. *J Gen Virol*. 2017; 98(12):2912-2913. [DOI:10.1099/jgv.0.000959] [PMID]
- [3] Bracken MK, Hayes BC, Kandel SR, Scott-Shemon D, Ackerson L, Hoffman MA. Viral protein requirements for assembly and release of human parainfluenza virus type 3 virus-like particles. *J Gen Virol*. 2016; 97(6):1305-1310. [DOI:10.1099/jgv.0.000452] [PMID]
- [4] Makoschey B, Berge AC. Review on bovine respiratory syncytial virus and bovine parainfluenza—usual suspects in bovine respiratory disease—a narrative review. *BMC Vet Res*. 2021; 17(1):261. [DOI:10.1186/s12917-021-02935-5] [PMID]
- [5] Franklyne JS, Gopinath PM, Mukherjee A, Chandrasekaran N. Nanoemulsions: the rising star of antiviral therapeutics and nanodelivery system—current status and prospects. *Curr Opin Colloid Interface Sci*. 2021; 54:101458. [DOI:10.1016/j.cocis.2021.101458] [PMID]
- [6] Jamshidi-Kia F, Lorigooini Z, Amini-Khoei H. Medicinal plants: past history and future perspective. *J Herbmed Pharmacol*. 2018; 7(1):1-7. [DOI:10.15171/jhp.2018.01] [PMID]
- [7] Lelešius R, Karpovaitė A, Mickienė R, Drevinskas T, Tiso N, Ragažinskienė O, et al. In vitro antiviral activity of fifteen plant extracts against avian infectious bronchitis virus. *BMC Vet Res*. 2019; 15(1):178. [DOI:10.1186/s12917-019-1925-6] [PMID]
- [8] Souto EB, Severino P, Marques C, Andrade LN, Durazzo A, Lucarini M, et al. Croton argyrophyllus Kunth essential oil-loaded solid lipid nanoparticles: evaluation of release profile, antioxidant activity and cytotoxicity in a neuroblastoma cell line. *Sustainability (Basel)*. 2020; 12(18):7697. [DOI:10.3390/su12187697] [PMID]
- [9] de Sousa DP, Damasceno ROS, Amorati R, Elshabrawy HA, de Castro RD, Bezerra DP, et al. Essential oils: chemistry and pharmacological activities. *Biomolecules*. 2023; 13(7):1144. [DOI:10.3390/biom13071144] [PMID]
- [10] Pilau MR, Alves SH, Weiblen R, Arenhart S, Cueto AP, Lovato LT. Antiviral activity of the *Lippia graveolens* (Mexican oregano) essential oil and its main compound carvacrol against human and animal viruses. *Braz J Microbiol*. 2011; 42(4):1616-1624. [DOI:10.1590/S1517-83822011000400049] [PMID]
- [11] Weber ND, Andersen DO, North JA, Murray BK, Lawson LD, Hughes BG. In vitro virucidal effects of *Allium sativum* (garlic) extract and compounds. *Planta Med*. 1992; 58(5):417-423. [DOI:10.1055/s-2006-961504] [PMID]

- 620 [12] Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of
621 selected monoterpenes derived from essential oils. *Phytother Res.* 2010; 24(5):673-679.
622 [DOI:10.1002/ptr.2955] [PMID]
- 623 [13] Benencia F, Courrèges MC. Antiviral activity of sandalwood oil against herpes simplex
624 viruses-1 and -2. *Phytomedicine.* 1999; 6(2):119-123. [DOI:10.1016/S0944-7113(99)80046-
625 4] [PMID]
- 626 [14] He W, Zhai X, Su J, Ye R, Zheng Y, Su S. Antiviral activity of germacrone against
627 pseudorabies virus in vitro. *Pathogens.* 2019; 8(4):258. [DOI:10.3390/pathogens8040258]
628 [PMID]
- 629 [15] Yang PL. Metabolomics and lipidomics: Yet more ways your health is influenced by fat.
630 In: Katze MG, Korth MJ, Law GL, Nathanson N, editors. *Viral Pathogenesis.* Boston:
631 Academic Press (Elsevier); 2016. p. 181-198. [DOI:10.1016/B978-0-12-800964-2.00014-8]
632 [PMID]
- 633 [16] Franklyne JS, Iyer S, Ebenazer A, Mukherjee A, Chandrasekaran N. Essential oil
634 nanoemulsions: antibacterial activity in contaminated fruit juices. *Int J Food Sci Technol.*
635 2019; 54(9):2802-2810. [DOI:10.1111/ijfs.14195] [PMID]
- 636 [17] Ghaderi L, Aliahmadi A, Ebrahimi SN, Rafati H. Effective inhibition and eradication of
637 *Pseudomonas aeruginosa* biofilms by *Satureja khuzistanica* essential oil nanoemulsion. *J*
638 *Drug Deliv Sci Technol.* 2021; 61:102260. [DOI:10.1016/j.jddst.2020.102260] [PMID]
- 639 [18] Moradi Alvand Z, Parseghian L, Aliahmadi A, Rahimi M, Rafati H. Nanoencapsulated
640 *Thymus daenensis* and *Mentha piperita* essential oil for bacterial and biofilm eradication
641 using microfluidic technology. *Int J Pharm.* 2024; 651:123726.
642 [DOI:10.1016/j.ijpharm.2024.123726] [PMID]
- 643 [19] Mazarei Z, Rafati H. Nanoemulsification of *Satureja khuzestanica* essential oil and pure
644 carvacrol: Comparison of physicochemical properties and antimicrobial activity against food
645 pathogens. *LWT.* 2019; 100:328-334. [DOI:10.1016/j.lwt.2018.10.094] [PMID]
- 646 [20] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to
647 proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65(1-2):55-63.
648 [DOI:10.1016/0022-1759(83)90303-4] [PMID]
- 649 [21] DIN EN 14476:2013+A1:2015. Chemical disinfectants and antiseptics – Quantitative
650 suspension test for the evaluation of virucidal activity in the medical area – Test method and
651 requirements (Phase 2/Step 1). European Committee for Standardization; 2015. [DOI]
652 [PMID]
- 653 [22] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am J*
654 *Epidemiol.* 1938; 27(3):493-497. [DOI:10.1093/oxfordjournals.aje.a118408] [PMID]
- 655 [23] Thonur L, Maley M, Gilray J, Crook T, Laming E, Turnbull D, Nath M, Willoughby K.
656 One-step multiplex real-time RT-PCR for the detection of bovine respiratory syncytial virus,
657 bovine herpesvirus 1 and bovine parainfluenza virus 3. *BMC Vet Res.* 2012; 8:37.
658 [DOI:10.1186/1746-6148-8-37] [PMID]

- 659 [24] Mehta SK, Kaur G. Microemulsions as carriers for therapeutic molecules. *Recent Pat*
660 *Drug Deliv Formul.* 2010; 4(1):35-48. [DOI:10.2174/187221110789957563] [PMID]
- 661 [25] Yousefzadi M, Riahi-Madvar A, Hadian J, Rezaee F, Rafiee R, Biniiaz M. Toxicity of
662 essential oil of *Satureja khuzistanica*: In vitro cytotoxicity and antimicrobial activity. *J*
663 *Immunotoxicol.* 2014; 11(1):50-55. [DOI:10.3109/1547691X.2013.793401] [PMID]
- 664 [26] Luo R, Dong Y, Fang F. [Article in Chinese]. *Zhonghua Shi Yan He Lin Chuang Bing*
665 *Du Xue Za Zhi.* 2001; 15(2):135-138. [DOI] [PMID]
- 666 [27] Moghimi R, Ghaderi L, Aliahmadi A, McClements DJ, Rafati H. Development of
667 antimicrobial nanoemulsion-based delivery systems against selected pathogenic bacteria
668 using a thymol-rich *Thymus daenensis* essential oil. *J Appl Microbiol.* 2017; 123(3):832-840.
669 [DOI:10.1111/jam.13521] [PMID]
- 670 [28] Moradi Alvand Z, Rahimi M, Rafati H. Interaction of a natural compound nanoemulsion
671 with Gram-negative and Gram-positive bacterial membrane: A mechanism-based study using
672 a microfluidic chip and DESI technique. *Int J Pharm.* 2022;626:122181.
673 [DOI:10.1016/j.ijpharm.2022.122181]
- 674 [29] Nickavar B, Mojab F, Dolat-Abadi R. Analysis of the essential oils of two *Thymus* species
675 from Iran. *Food Chem.* 2005;90(4):609-611. [DOI:10.1016/j.foodchem.2004.04.020]
- 676 [30] Alizadeh A, Alizadeh O, Amari G, Zare M. Essential oil composition, total phenolic
677 content, antioxidant activity and antifungal properties of Iranian *Thymus daenensis* subsp.
678 *daenensis* Celak. as influenced by ontogenetical variation. *J Essent Oil Bear Plants.*
679 2013;16(1):59-70. [DOI:10.1080/0972060X.2013.764176]
- 680 [31] Motlhatlego KE, Mehrbod P, Fotouhi F, Abdalla MA, Eloff JN, McGaw LJ. Anti-
681 influenza A virus activity of two *Newtonia* species and the isolated compound myricetin-3-O-
682 rhamnoside. *BMC Complement Med Ther.* 2021;21(1):92. [DOI:10.1186/s12906-021-03274-
683 0] [PMID]