Nanomed. J. 9(1): 43-56, Winter 2022

RESEARCH PAPER

Fabrication, characterization and evaluation of anti-cancer and antibacterial properties of nanosystems containing Hedera Helix aqueous extracts

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ABSTRACT

Objective(s): In this study we synthesized and characterized nanoniosome containing Hedera Helix extract to evaluate its therapeutic properties on breast cancer cells in order to provide a new effective treatment strategy with low side effects for the treatment of this malignancy in the clinical stage.

Materials and Methods: After extracting the Hedera helix by Soxhlet method, different formulations of noisomes containing the extract were synthesized and after investigating the load and release rate of the drug by spectrophotometry, the optimal formulation was selected. Then other physiochemical properties of nanosystems such as size and zeta potential by DLS method, system interaction and extract by FTIR method, system response to temperature and pH stimuli by spectrophotometry, nanoparticle morphology using SEM and AFM microscope, the stability of nanoparticles over 6 months and the antimicrobial properties of the system compared to the free form of extract were examined.

Results: Finally, the cellular uptake of nanosystem by flow cytometry microscope and its toxicity on MCF-7 and BT-474 breast cancer cell lines, MCF-10A breast normal cell line and HFF cell line were evaluated by MTT method. Results showed that the synthesized nanosystems with a size of 75.1 nm and PDI of 0.345 with a zeta potential of -20.6 ± 0.44 mV were morphologically suitable and had no interaction between niosomes and extracts. Also, the Encapsulation Efficiency in the system was 80.1 ± 2.2% and the drug release rate from the nanosystem was 57.2% in 72 hours.

Conclusion: The stability of the system during 6 months and the response of the system to various stimuli was also appropriate. Also, encapsulation of the extract not only improved its antimicrobial properties compared to the extract, but also increased its anti-cancer effect on cancer cells although the nanosystem had no toxic effect on normal cells.

Keywords: *Anti-cancer, Hedera Helix Nanosystems, Nanoniosome, Nanotechnology*

How to cite this article

Akhlaghi M, Taebpour M, Sharafaldini M, Javani O, Haghiralsadat BF, Orojalian F, Shadani Sh, Tofighi D. Fabrication, characterization and evaluation of anti-cancer and antibacterial properties of nanosystems containing Hedera Helix aqueous extracts. Nanomed J. 2022; 9(1): 43-56. DOI: 10.22038/NMJ.2022.62364.1647

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Note. This manuscript was submitted on October 10, 2020; approved on December 20, 2021

INTRODUCTION

Breast cancer is the most common cancer and also the leading cause of cancer death among women. According to the Global Cancer Statistics (GLOBOCAN),

breast cancer is the second most prevalent cancer in the world after lung cancer (1). Annually, more than 1.5 million women are diagnosed with breast cancer worldwide, which is about 25% of all cancers in women. Breast cancer is also accounted for more than 450,000 deaths yearly (2). According to the World Health Organization, the incidence rate of breast cancer is expected to increase to 3.2 million new cases by 2050 (3). Although breast cancer occurs in men, it is 100 times more common in women than in men. Breast cancer that develops in breast cells is a type of metastatic cancer that can usually spread to distant organs such as the heart, brain, bone, and lungs, which is a major cause of its incurability (2). Prognosis and early diagnosis of breast cancer is the most important factor in the treatment of this malignancy. In addition to tumor resection, chemotherapy and radiotherapy are also important strategies in the treatment of breast cancer. Docetaxel (DCX), Thioridazine (THZ), Disulfiram (DSF), Camptothecin (CPT) are some of the most common chemotherapy agents that used in the treatment of breast cancer (4). Resistance of cancerous cells to chemotherapy agents is one of the barriers to using chemotherapy in the treatment of breast cancer. Also, the inability of differentiation cancer cells from healthy cells and the adverse effect on non-target tissue by chemotherapeutic agents are among other challenging factors in chemotherapy. The short half-life of chemotherapy agents in the body's circulatory system and their scavenging by macrophages on the other hand, made breast cancer treatment arduous. The poor solubility of these drugs in water also causes problems in their uptake from biological membranes of cells and by adding of the general adverse side effects of chemotherapy to these challenges, the need for a new strategy in order to breast cancer treatment is highlight (5, 6). Nanotechnology tries to improve the delivery of anti-cancer agents to cancer cells by designing new drug delivery systems by using low-biotoxicity materials and easy biodegradability, and solves one of the biggest problems in 21st century by introducing a new efficient treatment system (7-10). Meanwhile, the use of herbs and their extracts and essential oils as anti-cancer agents has attracted the attention of researchers. Using of herbs as therapeutic agents has roots in human history and in various types of traditional medicine, herbs had been used extensively to treat various diseases (11). Nowadays also, using of medicinal plants is sharply increasing in all over the world due to their natural nature, compatibility with the human body, low side effects and reasonable price (12). Ivy is among these herbs. Ivy, scientifically known as Hedera Helix, belongs to the Araliacease family and is among plants with therapeutic properties. This plant is one of the evergreen plants that is found in most parts of the world. There have been many studies on the therapeutic effects of Ivy. For example, the leaves of lvy have been proven to have anti-inflammatory and anti-microbial properties and has also been demonstrated that is useful in the treatment of diabetes. Ivy has also been used in respiratory disease treatment such as asthma and bronchitis. Photochemical analysis of this plant has shown that the extract of this plant contains effective ingredients such as tannins, saponins, alkaloids and flavonoids, whose antioxidant and anti-cancer properties can be due to the presence of these chemical compounds in its chemical construction (13-15).

As mentioned, the statistics show the inefficiency of the current treatment methods in breast cancer treatment which in turn, clarify the basic need for a safe, consistent and effective drug delivery system. One of the strategies studied in the designing novel drug delivery system is synthesizing nanovesicles as drug delivery carriers such as liposomes and niosomes. Niosomes were first introduced in the cosmetics industry in 1970 and then their use in medicine was evaluated. Niosome, an enclosed bilayer structure, is lipid carrier that is formed from the accumulation of non-ionic surfactants in the aqueous medium and due to presence of cholesterol and its derivatives in its chemical structure, has the ability to transport hydrophilic and hydrophilic drugs. The easy and low cost design, flexibility and appropriate biodegradability, slow drug release and non-immunogenicity are among the advantages of using noisome compared to liposome (16, 17). So, the aim of this study was to synthesized nanoniosomes to deliver Hedera helix extract as an anti-cancer agent with low side effects, to breast cancer cells and characterized its physicochemical and antimicrobial properties in order to achieve an effective and optimal formulation to improve the treatment of breast cancer so that may be able to reduce the problems caused by this highly prevalent disease in the future.

MATERIALS AND METHODS

Material

Tween-60 and Span-60 were provided

from DaeJung Chemicals & Metals, South Korea and DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphocholine phospholipid) also was purchased from Ludwigshafen, Germany. Cholesterol, PBS tabs, dialysis bags (MW=12 kDa), DMSO (dimethyl sulfoxide), MTT (3 – (4, 5-dime-thylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), paraformaldehyde and fetal bovine serum (FBS) were procured from Sigma, USA and Isopropyl buffer also purchased from Merck, Germany. All cell lines and bacterial and fungal colonies used in this study were prepared from Pasteur Institute of Tehran and all of cell cultures and microbial cultures were performed according to ATCC and MTCC protocols, respectively. penicillin–streptomycin was also supplied from Gibco, Grand Island, NY. DAPI (40, 6-diamidino2-phenylindole) and DIL Stain (1, 10-Dioctadecyl-3,3,30,30-Tetramethylindocarbocyanine Perchlorate) were obtained from Thermo Fisher Scientific (Waltham, MA). penicillin–streptomycin tabs also were provided from Gibco, Grand Island, NY. All further chemicals, salts and solvents used in this study were of the analytical grade and used without any catharsis unless determined.

Extraction of Hedera helix (HH)

After preparing the Ivy, its type and species were approved by botanical experts of Yazd University. Then, the plant was dried away from sunlight at room temperature, and then was powdered using an electric grinder. Extraction of the plant was done by Soxhlet method. The Soxhlet extractor consists of 4 parts: heater, balloon, extraction chamber and condenser. First, 50 g of the obtained powder was squeezed into the cartridge case and then placed inside the soxhlet column. Then we poured 500 ml of 70% ethanol into the balloon and connected the soxhlet to it. Finally, by installing a condenser and water inlet and outlet extraction was performed.

Determination of the wavelength maximum absorbance (λmax) and plotting standard curves

Spectrophotometry was used to determine the λmax of HH extract. In this method, first stock solution of HH extract with a concentration of 1 mg/mL in Phosphate-buffered saline (PBS), (Sigma, USA) and isopropyl buffer (Merck, Germany) was prepared. Then, using stock solution, different concentrations of the extract (500, 250, 125, 62.5, 30, 15 and 7.5 mg/mL) were prepared by using dilution method in PBS and isopropyl solvents. The absorption spectrum was then obtained by a spectrophotometer (Epoch, USA) in the wavelengths range of 200 to 800 nm for all samples. The wavelength maximum absorbance was the wavelength at which the highest amount of absorption was obtained at all concentrations. Then, using the absorbance amounts obtained from different concentration at the λmax, the standard curve of HH extract in PBS and isopropyl buffer was plot and its linear equation was calculated. The experiments were performed at this stage with three repetitions.

Preparation of niosomal nanocarriers

Niosomes containing HH extract (Nio-HHE) were synthesized according to Table 1. In order to achieve optimal formulation and ensure optimal results following processes were fulfilled:

- Investigating the effects of type of surfactant applied and different molar ratios non-ionic surfactants: cholesterol was performed on synthesized nanocarriers.
- Optimal formula was selected based on best condition of encapsulation efficiency (EE%) and drug release rate (%) from nanoparticles.
- 20% DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphocholine phospholipid) was added to nanoniosome in order to cause temperature sensitivity in nanoniosomes
- In order to enhance stability of nanoparticles in blood circulation and influence of HH extract bioavailability, 5% DSPE-PEG (distearoyl phosphoethanolamine-polyethylene glycol) were added and its, effects on mentioned parameters were evaluated.

Nio-HHE were prepared by the thin film

Table 1. Molar ratios of ingredients of niosomal formulations

Formula	Tween60 (%)	Span60 (%)	Cholesterol (%)	DPPC (%)	DSPE: PEG (%)
F1	0	75	25	0	0
F2	75	0	25	0	0
F3	0	75	25	20	0
F4	75	0	25	20	0
F5*	75	0	25	20	5

method. Briefly, various ratios of non-ionic surfactants (Tween-60, Span-60) (DaeJung Chemicals & Metals, South Korea), cholesterol (Sigma, USA) and DPPC (Ludwigshafen, Germany) according to Tab 1, were dissolved in chloroform. The thin film method has two phases, organic and aqueous. In the organic phase, the cholesterol and surfactants were first dissolved in a round bottom balloon using chloroform solvent. The solvent after 20 min was removed by using a rotary instrument (Heidolph, Heivap); (50 ° C, 150 rpm) and vacuum conditions. After chloroform evaporates, a thin lipid film forms at the end of the balloon. In order to solvent completely remove, the balloon was aerated with nitrogen gas for a few seconds and kept at 4 ° C for 24 hr. Loading of the drug in niosomes was done inactively in the aqueous phase. For this purpose, the thin film was hydrated by rotary instruments (temperature 60 ° C, rpm 150) for 60 min using HH extract with 2 mg/mL solution dissolved in PBS buffer (pH = 7). For reducing the size of the niosomes, microtipe probe sonicator (ChromeTech, UH1200B) (60% power, 10 seconds on, 15 seconds off) was used for 45 min. To prevent the sample temperature rising, the niosomal solution was placed in a container containing ice. Thereupon, by using dialysis bag which had cutoff 12 kDa, non-capsulated and free HH extract was separated from Nio-HHE.

Evaluation of encapsulation efficiency of niosomes (EE%)

To figure out the encapsulation efficiency of nanosystem, first, unentrapped extract had to be removed. For this purpose, the separation method was used by dialysis bag at 4 °C. Then, the synthesized niosomes were lysed with isopropanol. The use of isopropanol caused the membrane of niosomes be broken and the Hedera Helix extract released. Afterwards, the sample absorbance was read spectrophotometer (Epoch, USA) at λmax of HH extract and the concentration of encapsulated HH extract were calculated by standard curve of HH extract in isopropanol and Equation 1:

Equation1:

EE%: encapsulated extract in niosomes (mg/mL)/ total drug (mg/mL) × 100

Determination of size, dispersion index (PDI) and zeta potential of nanoniosome

The size, zeta potential and PDI of nanoparticles were determined using dynamic laser scattering (DLS), by Brookhaven Instruments (Holtsville, NY). Nanoniosomes were analyzed by radiation of laser light in 657nm at a 90̊ angle and 25 °C. Also, the samples were analyzed in 5 repetitions and each time with a duration of 30 seconds. To determine the size, 600 µL of samples with a concentration of 0.5 to 0.1 mg/mL were used. Zeta potential / surface charge of nanosystems was determined at room temperature using 1500 µL of samples with a concentration of 0.1 mg/mL.

Morphological characterization

The best way to ensure the shape and structure of nanoparticles is microscopic observation. In this study, atomic force microscope (AFM) (Nanowizard II; JPK instruments; Germany) and scanning electron microscope (SEM) (EM3200-KYKY, China) were used to examine the morphology of nanoniosomes. Nanoparticles were examined for roughness, shape and uniformity. AFM is a microscope for observing samples with nanometer dimensions and examining their surface topography. For imagining of nanoparticles by AFM, niosomes were diluted to 1;1000 with water and after 20 min sonication, samples were placed on a mica sheet and observed. In order to prepare the sample for imagining by SEM, a certain amount of Nio-HHE was placed on the glass plate and dried in the presence of air and away from sunlight, and finally coated with a thin layer of gold.

Functional groups characterization

FTIR spectroscopy technique was used to investigate interaction between the extract and the drug carrier. The peaks obtained in the IR spectrum represent the sample functional groups. Thus, the FTIR spectrum of the Nio-HHF and blank niosome were obtained with the FTIR spectrophotometer. For this purpose, first 1 mg of each sample was added in a ratio of 1 to 100 to the potassium Bromide (KBr) and was compressed by hydraulic press at a pressure of 5 to 8 tons per centimeter. The compressed cube was placed on a plate with a thickness of about 0.1 cm and each sample was examined at range of 400-4000 cm^{-1} by FT-IR spectrophotometer (Brucker, Germany) and Its functional groups was identified.

Evaluation of the stability of noisome containing Hedera helix extract

The stability of the Nio-HHE in terms of electric charge, encapsulation rate, size and dispersion index was investigated. For this purpose, Nio-HHE after synthesis, was kept at 4 ° C for 180 days and at different time intervals, their size, electric charge and PDI were investigated by DLS method. Also,

the encapsulation rate of Nio-HHE was examined in order to investigate the leakage of the extract from the niosomes by spectrophotometric method according to what was previously described.

Evaluation of antimicrobial activity of Nio-HHE in comparison with free form of extract

20 µl of blank niosomes, HH extract and Nio-HHE were placed on a TSA plate containing 107 cell/mL of Staphylococcus aureus and Escherichia coli bacteria and plate was incubated at 37 °C for 24 hr. Ampicillin disc was also used as a control for antimicrobial activity. Then, the non-growth zone around the samples were used to study the antimicrobial activity of the extract and Nio-HHE.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

MIC is actually the lowest concentration of an antimicrobial agent that can inhibit the growth of microorganisms. In this study, sequential dilution technique was used to evaluate the MIC of the nanosystem. For this purpose, 11 test tubes were used which each test tube contains different concentrations of the antimicrobial agent and the same amount of microorganism. At first, 0.5 ml of culture medium was added to tubes 2 to 11. Then, 2.5 ml sample was added to tubes 1 and 2, and then 0.5 ml tube 2 was transferred to tube 3 and this process continued until tube 9 and finally 0.5 ml of tube 9 was removed and discarded. This process was performed for Nio-HHE and HH extract separately. Then 0.5 ml of Escherichia coli bacterial suspension and of Staphylococcus aureus were inoculated into each of 2 to 10 tubes. MBC also refers to the lowest concentration of an antimicrobial agent that can kill 99.9% of the population of inoculated microorganisms. In the standard method, after determining the MIC, 100 µL of suspension was separated from each tube in which the growth of microorganisms was not observed and then was cultured on a TSA plate. After that, it was incubated for 24 hr at 37 ° C and the number of grown colonies was counted. The concentration at which the number of colonies decreases to one-thousandth of the amount of the initial suspension is reported as the MBC concentration.

Antifungal Analysis

20 µl of sample (extracts and Nio-HHE) were added to Sabouraud dextrose broth as a liquid culture medium and Sabouraud dextrose agar as a solid culture medium containing 107 cells/ml of Candida albicanse, Trichophyton.rubrum and Trichophyton mentagrophytes and incubated for 24 hr at 37 ° C. To show antifungal activity for each concentration of antifungal agent, the non-growth area around the sample was evaluated. The ampicillin antibiotic disc was used as a control group(10, 18).

In vitro thermo- and pH-sensitive HH extract release assay

To investigate the extract release pattern from niosomes, 1 mL of the niosomal solution was poured into two separate dialysis bags and transferred to a falcon tube containing 10 mL of PBS buffer. The falcons were then stirred under two different temperatures (37, 42 °C) and different pH conditions (pH=4.5, 5.2, 7.4) in order to evaluate the response of nanosystem to stimuli. Sampling of PBS medium around dialysis bags was performed at 1, 2, 3, 4, 6, 8, 12, 24 and 48 hr and the volume of each sample was replaced with the same volume, temperature and pH of fresh buffer simultaneously. Then, the absorption of the samples was read using a spectrophotometer at λmax of HH extract and the release rate of extract from the system was calculated for each of the temperature and pH conditions at different times using the standard curve of extract in PBS.

Cell culture

Breast cancer cell lines MCF-7 and BT-474 as cancerous cell lines and also MCF-10A cells as healthy breast cell line were purchased from Pasteur Institute (Tehran, Iran) and also human foreskin skin cell line (HFF) as normal cell line were obtained from Stem cell biology research center (Yazd, Iran). Cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) (Sigma, USA) and penicillin–streptomycin (Gibco, Grand Island, NY) and all cells incubated in an incubator at 37 ° C, 95% humidity and 5% CO2 (standard condition).

Cellular uptake

The cellular uptake of HH extract and Nio-HHE were investigated in MCF-7 and HFF cells lines using fluorescence microscopy. Cells were seed onto six walls plates (1.5×105 cells/per well) and after that, treated with HH extract and Nio-HHE for 6 hr. Then, cell rinsed with PBS buffer for three times and fix with 4% paraformaldehyde solution (Sigma, USA). The nuclei of the cells were

counterstained with DAPI (1 mg/mL) for 15 min and imaged with a fluorescence microscope (BX61, Olympus, Japan). Hedera helix extract was obtained fluorescent property by adding 0.1% mol of Dil.

MTT assay

MTT assay was used to evaluate the toxicity of the HH extract and Nio-HHE on cancer and normal cells. The basis of the MTT method is the reduction of tetrazolium by mitochondrial reductase enzymes. At first, cells were cultured in 96 plates for 48 hr. After reaching the required number of cells (1×10^4 cell/per well), the cells were treated for 48 hr at various concentrations (1000, 750, 500, 250, 125, 62.5 and 31.25) of HH extract, blank niosomes and Nio-HHE. Then 20 µL of MTT salt with a concentration of 0.5 µl was added to each well and incubation was performed for 4 hr. The supernatant was then removed and 150 µL of Dimethyl sulfoxide (DMSO) (Sigma, USA) was added to each well in order to remove Formazon crystals, and incubation was performed for another 30 min. After that, the absorption of wells at wavelength of 570 nm was read using ELISA reader (Synergy HTX, Bio Tek, USA) and the survival rate of cells was calculated. To evaluate the non-toxicity synthesized niosomes on normal body cells, different dilutions of blank niosome were applied on HFF cells for 48 hr and then the toxicity of synthesized nanosystems on normal cells was calculated according to the mentioned method.

Statistical analysis

Data analyzed by GraphPad PRISM version 8 (GraphPad, San Diego, CA). All results were collected with three repetitions and the values were display as the mean ± standard deviation. A Students t-Test and ANOWA test were used for comparing two independent groups and multiple groups respectively.

RESULTS

Maximum wavelength absorbance and standard curves

By evaluation of absorbance spectrum at various concentration of HH extract obtained from spectrophotometry at range of 200 to 800 nm, could be conclude the maximum absorbance of HH extract is at wavelength of 320 nm (Fig 1A). Also, Fig 1B and C, illustrate the standard curves of HH extract in PBS and Isopropanol buffers respectively.

Optimal formul

Niosomes containing Hedera Helix extract were prepared with various molar ratio of surfactant; cholesterol and with or without DPPC or DSPE: PEG (%) according to Tab 1. So, in order to select optimal formula, all synthesized niosomal formulation were evaluated base on their size, EE% and maximum extract release in 72 hr. EE% and drug release are among most important factors in selection of drug carrier to deliver drug to target tissue. According to Table 2, F5 formula selected as optimal formula because in addition to having a suitable EE% (80.1±2.2%) compared to other formulations, it has also shown a proper release of the drug at 72 hr (57.2%) compared to other niosomal formulations and other analyzes were performed on it. To evaluate the effect of DSPE-mPEG2000 on niosomes stability, 5% of DSPE-mPEG2000 was added to the selected formulation F5.

Size, Polydispersity index (PDI) and Zeta potential

According to Fig. 2A, the size of optimal formula after HHE loaded and after added DSPE-PEG was 75.1 nm and the PDI was 0.345. The average size of nanoparticles is less than 100nm which make them easily pass through the blood barriers. DSPE-PEG because of its negative charge, reduces the size of nanoparticles by repulsion forces and prevents them from accumulating (19). In this study, after

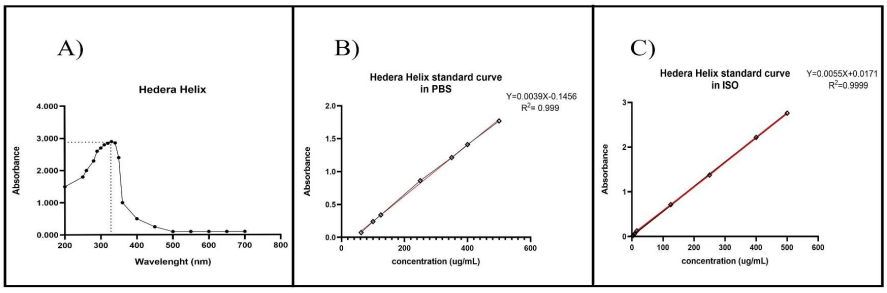

Fig 1. A) Absorption spectrum of HH extract at wavelengts of 200 to 800 nm. The maximum absorption value is at 320nm. B) The standard curve of HH extract in PBS buffer.The equation line is Y=0.0039X-0.1456 and squared value (R2) is 0.999. C) The standard curve of HH extrat in isopropyl. The equation line is Y=0055X+0.0171 and squared value (R2) is 0.9999.

Table 2. Effects of various molar ration of non-ionic surfactant; cholesterol and phospholipids DPPC and DSPE: PEG (%) on EE% and % drug release. F5 selected as optimal formula

Formula Code	Encapsulation Efficiency (% EE Mean± SD)	%Release (24h)	%Release (48h)	%Release (72h)
F1	35±2.2	33.7	36.7	41.6
F2	44±2.3	32.7	35.4	36.2
F3	62±1.4	43.5	45.4	51.1
F4	74±0.5	50.1	54.4	57.6
F5*	80.1±2.2	48.2	53.1	57.2

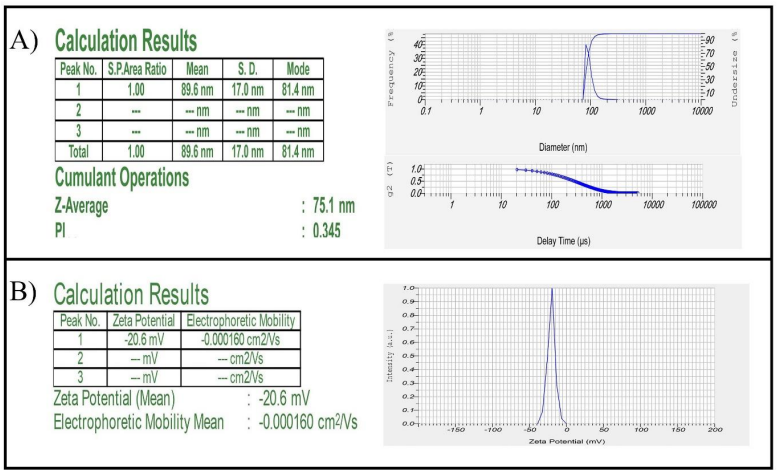

Fig 2. A, the average size of nanoparticles after loading of HH extract and adding DSPE-PEG obtained from DLS. B, the average zeta potential (mV) of Nio-HHE after adding DSPE-PEG obtained from DLS

adding DSPE-PEG, the nanoparticle size was reduced from 80.32 to 75.1. Fig 2B demonstrate that the Nio-HHE were anionic type and their zeta potential was -20.6 ± 0.44 mV. This negative charge reduces the toxicity of the system and improves its performance by preventing the accumulation and deposition of nanoparticles. Table 3, shows the size, zeta potential, EE% and % drug release of optimal formula after adding DSPE-mPEG.

Morphological assay

The morphology of nanoparticle was characterized by AFM and SEM (Fig 3A, B). The findings of these analysis confirm the DLS results. The images comprehensibly indicate the spherical shape of nanoparticles. The nanoniosomes also have uniform structure and smooth surface. No accumulation is observed in them.

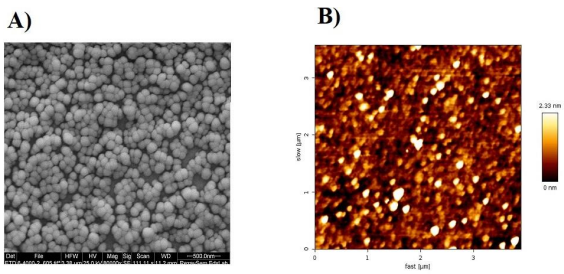

Fig 3. Morphological evaluation. A, scanning electron microscopy of noisome containing hedera helix extract. B, atomic forces microscopy of noisome containing hedera helix extract

Evaluation of interaction between extract and synthesized niosomes

FTIR technique was used to investigate the interaction of the nanosystem and the extract.

Table 3. Characterization of optimal formula F5

Formula Code	Encapsulation Efficiency (% EE Mean± SD)	%Release (72 h)	Size (nm)	PDI	Zeta potential (mv)
F5*	80.1±2.2	57.2	75.1	0.345	-20.6

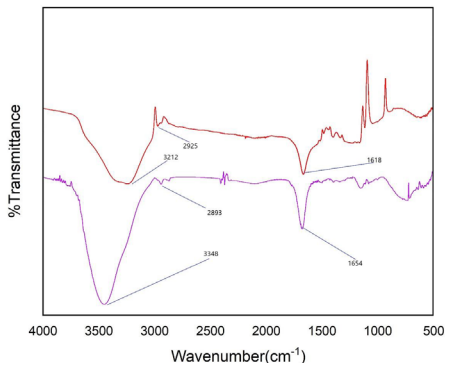

Fig 4. Optimal formula FTIR spectrum compare to blank niosome FTIR spectrum

According to the obtained spectra (Fig. 4), it is inferred that in the blank noisome the peak index is 3212 cm^{-1}, which indicates the alcoholic functional group (OH), which is the same peak with a slight difference of 3348 cm^{-1} is repeated in the Nio-HHE. The peak index of 2925 cm^{-1} is also evidence of the existence of an alkane functional group with C-H tensile bond, which is also repeated in the Nio-HHE with a slight difference at 2893 cm^{-1}. In general, by comparing the FTIR spectra, it can be concluded that the presence of the extract in the system did not cause remove or addition new peaks, which did not indicate the formation of a new structure or the decomposition of compounds in the system. Therefore, loading the extract in the system did not cause any abnormal interaction.

Evaluation of niosome containing hedera helix extract stability

Changes in surface charge, size, and PDI of optimal formula were monitored over 180 days. According to Fig. 5, the changes in nanoparticles size during the 6 months were very low. Also, the changes in the surface charge of nanoparticles have been very small and after 180 days, the particles have become slightly more positive. The rate of changes in the PDI of Nio-HHE after 180 days were rare either. Also, the loading rate of extract into the niosomse was examined after 180 days. According to the Fig. 5, it can be concluded that the leakage of nanoparticles was inconsiderable. In general, it can be concluded that the synthesized nanosystem was stable and changes in its physiochemical properties was negligible.

Evaluation of antimicrobial activity of Nio-HHE

The lowest concentration of antimicrobial substance that prevents the growth of microorganisms is called MIC. Therefore, the clearest tube compared to the control group, which has the lowest concentration of antimicrobial agent, was identified and its concentration was reported. In this study, in order to investigate the

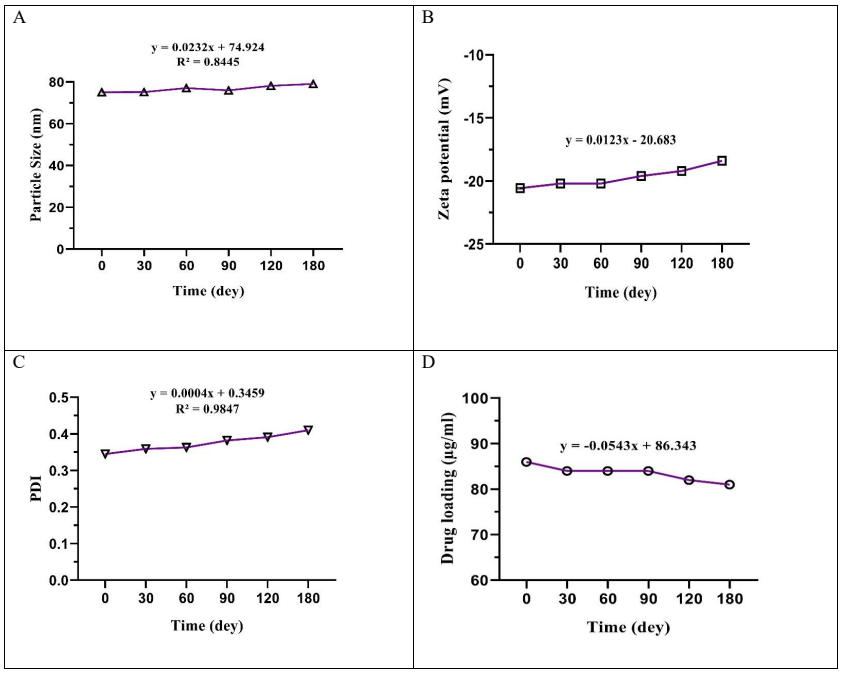

Fig 5. Evaluation of Nio-HHE stability during 180 days of storage at 4 ° C. A, Nanoparticle size changes. B, Nanoparticle zeta potential changes. C, PDI changes. D, extract loading rate changes

Table 4. The antimicrobial effect of Nio-HHE compare to free form of HH extract

Diameter of Zone of Inhibition (mm)	MBC (µg/ml)	MIC (µg/ml)	Antibacterial material type	Microorganism type
25	50	25	Hedera helix extract	Candida albicanse
28	25	12.5	Nio-HHE solution	
31	25	12.5	Hedera helix extract	Trichophyton.rubrum
34	12.5	6.25	Nio-HHE solution	
36	12.5	6.25	Hedera helix extract	Trichophyton.mentagrophytes
38	6.25	3.125	Nio-HHE solution	
10	50	25	Hedera helix extract	Escherchia.Coli bacteria
13	25	12.5	Nio-HHE solution	
22	25	12.5	Hedera helix extract	Staphyloccus.aureus bacteria
25	12.5	6.25	Nio-HHE solution	

turbidity of the tubes caused from nanovesicles, the spectrophotometric method at a wavelength of 600 nm was used. To determine MBC, 100 µL of all test tubes were removed and cultured, and after 24 hr of incubation at 37 °C, the plate had the most diluted colloidal solution and the number of colonies in that reached to 1/1000 of the initial colonies was considered as MBC. Table 4, also demonstrate the MBC, MIC and dimeter of zone of inhibition for mentioned bacteria and fungi. According to Table 4, the antimicrobial effect of Nio-HHE was increased compare to free form of HH extract.

Encapsulation efficiency and in vitro thermal- and pH-sensitive evaluation

Based on the above, the encapsulation efficiency (EE%) of niosomes for HH extract obtained using dialysis bag and isopropyl buffer was 80.1±2.2 (Table 2). Lower pH and higher temperature are among the differences in cancer cells compared to healthy cells, which is due to changes that occur in these cells. Since our nanocarriers respond to stimuli such as temperature and pH with differences in drug release (%), it can be concluded that, we designed temperature and pH-sensitive niosomes to increase efficiency of niosomal formula on cancer cells and reduce side effects on normal cells in order to enhance passive targeting of anti-cancer agents on cancerous cells. Fig. 6, show the *in vitro* release profile of HH extract from niosomes at various temperature and pH. By analyzing data, it could be concluded that fastest drug release from niosome occurred at pH=4.5 and temperature 42 °C that was

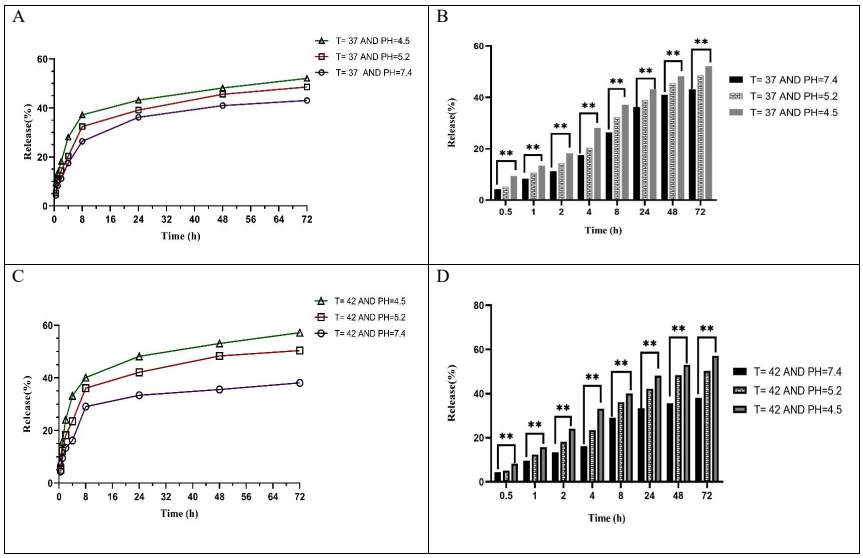

Fig 6. Different extract release pattern from nanoniosome in response to stimuli pH and temperature. A, HH extract release from at various pH in 37 ˚C. B, Differences in extract release rates from niosome at different times, in various pH at 37 ˚C. C, HH extract release from at various pH in 42 ˚C. D, Differences in extract release rates from niosome at different times, in various pH at 42 ˚C. **: *P*-value<0.05

57.2 in 72 hr, while the least release rate occurred in physiological condition (37° C and pH= 7.4) that was 38.1. Fig 6A, B, demonstrate different responses of synthesized nanosystems to temperature and pH stimuli. It can also be understood that the release of the extract from the nanoniosomes follows a two-phase pattern: a rapid release pattern (Due to differences in drug concentrations) in the first phase and a slow release pattern (Due to the reduction in drug concentration differences) in the second phase.

Cellular uptake

Cellular uptake of HH extract and Nio-HHE in MCF-7 and HFF cells shown in Fig 7. Images obtained by fluorescence microscopy demonstrate successful delivery of Nio-HHE to normal and cancerous cells. According to this data, the niosomes were impressively uptaked by the cells.

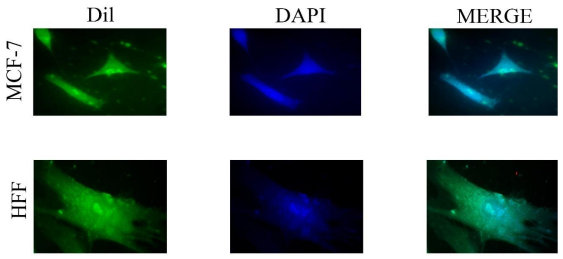

Fig 7. The cellular entry of Nio-HHE into MCF-7 cells as cancerous cells and HFF cells as normal cells were shown via cytoplasm staining (dial) and nucleus staining (depi). The integration of these images under a fluorescent microscope confirms the successful entry of the niosomal system into the nucleus of cells

Cytotoxicity study

As shown in Fig. 8, the cytotoxicity effect of HH extract was improved after encapsulation on cancerous cells. According to Fig 8, the MCF-7 and BT-474 breast cancer cells proliferation inhibited after 48 hr of treatment with Nio-HHE and free form of HH extract and this inhibition was stronger

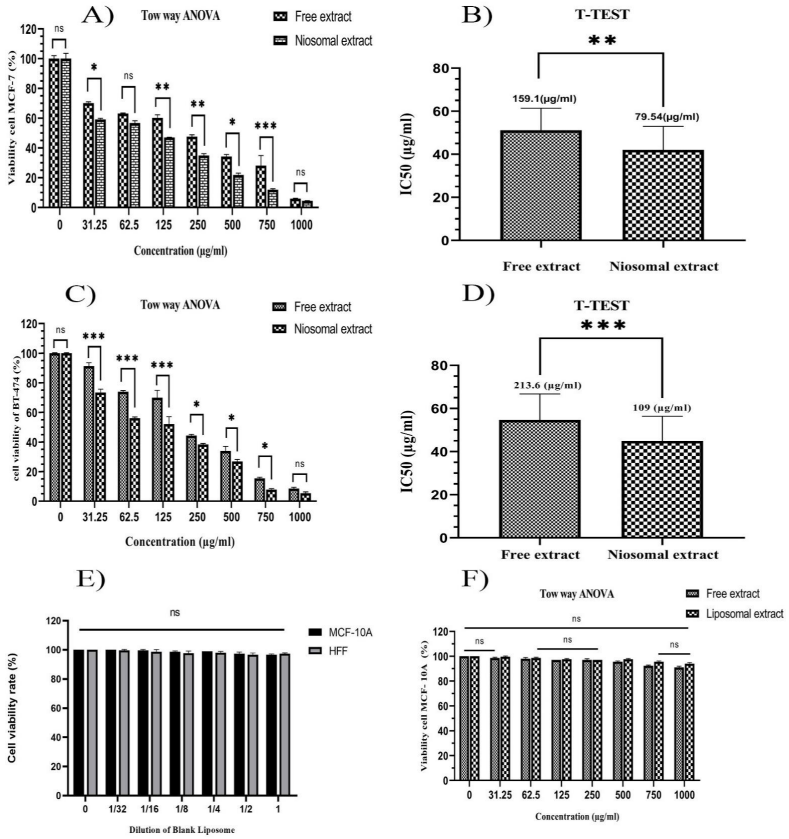

Fig 8. MTT assay results, A,Cytotoxicity of Nio-HHE and free HH extract on MCF-7 brrast cancer cell line. B, Comparsion between IC_{50} of Nio-HHE and free HH extract on MCF-7 cells. C, Cytotoxicity of Nio-HHE and free HH extract on BT-474 brast cancer cell line. D, Comparsion betwee IC_{50} of Nio-HHE and free HH extract on BT-474 cells. E, Cytotoxicity of blank niosome at various dilution on HFF and MCF-10A as normal cells. F, Cytotoxicity of Nio-HHE and free HH extract on MCF-10A as breast normal cell line. ns: no significant difference. *: *P*-value<0.05

in the presence of Nio-HHE compared to free of HH extract. Also MTT assay results showed that blank liposome had no toxicity on both normal and cancerous cells. More ever, the cytotoxicity effect of nanosystems on MCF-7 cell line is more than BT-474 which this may be due to better uptake of the Nio-HHE by MCF-7 cells. Nio-HHE and free of extract also had no toxic effect of MCF-10A and HFF cell lines as normal cells that was interesting observation. In all, the results showed that the mortality of breast cancer cell treated with Nio-HH increased significantly compared to free from of HH extract. This result also confirmed by comparing between IC_{50} value of Nio-HHE and free form of HH extract on cancer cells. Therefore, it can be concluded from the results of MTT assay that HH extract can inhibit the growth and proliferation of cancer cells, and by encapsulating it, its anti-cancer properties also increase.

DISCUSSION

Statistically, current treatments for breast cancer have not been effective. The resistance of tumor cells to chemotherapy and its severe side effects on the other hand, clarify a demand for a new treatment strategy. Many studies have suggested the use of herbs and their extracts as safe anti-cancer agents with low side effects to fight against cancer. In 2016, Gumushan - Aktas et al. in a study showed that Hedera helix extract could inhibit the growth and proliferation of prostate cancer cells in mice. They attributed the toxicity of HH extract on cancer cells to the presences compounds such as α-Hederin and hederagenin in the chemical structure of HH extract. α-Hederin can induce apoptosis in cancer cells (20). Barthomeuf et al in 2002, by isolating the active substances α-hederin, β-hederin and examining their cytotoxic effects on cancer cell lines and normal human fibroblast cells, concluded that these substances can inhibit the growth of cancer cells and also, can be toxic for fibroblast cells as normal cells. However, in order to cause cytotoxicity activity on normal cells, high concentrations of these substances were required in compared to cancer cells (21). In this study, while confirming the cytotoxicity effect of HH extract on breast cancer cells, it was shown that HH extract in low concentrations had no toxic effect on normal cells, which can be considered as an advantage in using of this extract as an anti-cancer agent. However, the use of herbs and their extracts by conventional methods faces challenges such as poor permeability, weak stability in the circulatory system and low solubility of these substances. Nanoscience tries to overcome these problems by introducing drug delivery nanocarriers to deliver these compounds to the target tissue and improve their therapeutic effects (12). Numerous studies have shown that niosomes can increase the stability of drugs in the circulatory system, increase their uptake by cancer cells, and generally improve their function. Encapsulation efficiency (EE%) is one of the important factors that is very important in the design and synthesizing of nanosystems and their selection as drug carriers. EE% related to various factors like type surfactant and ratio of surfactant/cholesterol and lipid/drug. The EE% of synthesized niosomes for HH extract in this study was 80.1±2.2. The large hydrophilic head group of Tween-60 compared to Span-60 causes the increasing in EE% and also, this hydrophilic head group has a higher chance for forming a hydrogen bond with the HH extract due to its phenolic group (22). In addition, the longer hydrophobic chain of Tween-60 than the Span 60 play a dominant role in HH extract entrapment (23). Excessive increase in cholesterol content can also reduce EE% by competing with lipophilic drugs to enter the bilayer and imbedded into nanoparticles that causes reduce the amount of drug encapsulation (24). Addition of phospholipids, including DPPC, can also increase EE% due to hydrogen bonding with the HH extract (25). According to the study of Li, Ya-Ping, et al by adding DSPE-PEG the EE % increased (increase aqueous space), addition of DSPE-PEG also increases the stability of nanoparticles in the circulatory system, enhance the solubility of hydrophobic drugs, and reduces the size of nanoparticles, which in turn, improve cellular uptake. So in general, by adding DSPE-PEG to drug nanocarriers, their anti-cancer activity be improved (26). As our study showed, the stability of the synthesized nanoparticles was appropriate in terms of leakage, size and charge, and the slight changes were negligible. So, this could be concluded that the optimal formula could overcome the niosome instability problems. The drug release also in another important factor for nanocarriers. The highest release rate of the HH extract from nanoniosomes in our study was 58% after 72 hr. Tween-60 can reduce drug leakage due to its long hydrophobic chain and thus reduce drug release rate and because of this chain that if the molar ratio of Tween-60 / cholesterol

increases, the drug release rate decreases. Increasing cholesterol content is also one of the factors that can enhance the drug release rate by interfering with the regular structure of the vesicle membrane. Adding DPPC also, due to the presence of unsaturated alkyl chain in its chemical structure, increases flexibility and mobility and thus increases drug release (27).

Drug release profile pattern and its response to stimuli is an important factor in niosomal system. Cancer cells have a lower pH and higher temperature compared to normal cells, which can be used them to overcome one of the most important barriers in chemotherapy, which is the lack of differentiation between cancer cells and normal cells. Among the stimuli that affect the release of the drug from the nanoniosome are the temperature, the structure of the niosome membrane and the pH of the surrounding buffer (28). In this study, we succeeded in synthesizing niosomes that were sensitive to temperature and pH and increased the rate of drug release from niosomes in response to this stimulus, which can be used to reduce the cytotoxicity of drugs on normal cells because cancer cells usually have higher temperatures and lower pH than normal cells. The results of this study demonstrated that the release rate of HH extract from the optimal formula at pH = 4.5 and the temperature of 37 ° C is more than 20% higher than the release rate of HH extract from the optimal formula in the physiological conditions (7.4 and the temperature of 37 ° C). Because at temperatures above 40 ° C, the membrane permeability of niosomes increases and as a result, the rate of drug release from the system also increases (29). Also, at more acidic pH, it creates a proton gradient across the membrane of the niosomes, which in turn increases the protonation of the amine group in the chemical composition of the HH extract, thereby increasing drug release (30). This is one of the factors that increase the cytotoxicity of encapsulated drugs compared to their free form. For example, Alemi et al. In 2017 showed that the anti-cancer effect of niosomes containing curcumin on MCF-7 cells is greater than its free form (31). In line with this study, our study also showed that the IC_{50}, the concentration of a substance that inhibits 50% cell growth, the Nio-HHE on MCF-7 and BT-474 cancer cells was 79.54 and 109 µg/mL, respectively. While the IC_{50} free form of the extract on those cells is 159.1 and 213.6 µg/mL, respectively.

The size of nanoparticles is another influential factor in their selection as drug carriers. The size of nisomes after loading of HH extract in their PDI were 75.1 nm and 0.345, respectively. The size of the niosomes effects on their distribution, elimination, stability of them on circulatory system, and therefore their capacity to targeting the target tissue. However, the chemical composition of niosomes plays a key role in using the appropriate method to reduce the size of niosomes. Numerous studies have shown that particle size is important in removing them from the body because the kidneys and spleen are able to filter particles with a size of less than 6 nm and less than 250 nm, respectively. The hepatic system has also been shown to be capable of removing particles in the range of 100 nm that the renal system has not filtered (32). So having a low PDI and the desired size are among the features that should be considered in the design of nanosystems. Several factors play roles in determining the size of niosomes, including the type of surfactant used to synthesize niosomes. Tween-60 has a lower hydrophobic-lipophilic balance (HLB) than other surfactants such as Span 60 because it has a lower hydrocarbon chain volume compared to the hydrophilic surface. It is also expected that in niosomes with same content of cholesterol, niosome with lower HLB value has smaller size (33). Cholesterol is also a factor that has a great impact on the size of nanoparticles. Numerous studies have shown that the addition of cholesterol causes increase in of the bilayer hydrophobicity of niosomes, resulting in a reduction in surface free energy and at the end causes reduction in nanoparticle size. However, the same studies have shown that the effect of cholesterol on the size of nanoparticles strongly depends on the type of surfactant used in their synthesis (34).

Zeta potential is also an important factor in the design of nanoparticles. Scientifically, although very positive and very negative values of zeta potential (more than 30) cause colloidal stability due to electrostatic repulsion, in practice, particles with different charge than body cells are quickly identified and eliminated by the body immune system. Negative zeta potential is also important because it inhibits the non-specific interactions with blood cells and thus reduces risk of scavenging by macrophages and also increases cellular uptake (35). According to a study by Zhang et al. in 2008, Nanoparticles with zeta potential

of -13 mV entered the MCF-7 (zeta potential -20 mV) cancer cells through endocytosis, although this was theoretically impossible due to electrical repulsion, but the uptake of nanoparticles with zeta potential in the range of zeta potential of cells is enhance by intervention of some proteins (36). The zeta potential of the nanoniosomes in this study was -20.6 mV, which was in the desired range and was close to the zeta potential of cancer cells, which led to better uptake of them by cancer cells, and images obtained by fluorescent microscopy also confirmed this claim.

Our main goal in this study was to improve the therapeutic effects of HH extract by encapsulating them into niosomes on breast cancer cells. Numerous studies have shown that encapsulation of active ingredients can improve their therapeutic function. For example, Hemmati et al. Showed in 2019 that by encapsulation of chemotherapy agents such as doxorubicin and quercetin into niosome, their therapeutic effect on gastric cancer cells increases compared to their free form (28). In 2017, Naderinezhad et al. Showed that by encapsulating chemotherapeutic agents, in addition to increasing their cytotoxic effect on cancer cells, they can reduce cell resistance to chemotherapy and also reduce unwanted side effects on normal cells (37). In this study, we succeeded in synthesizing slow release type and thermos-sensitive pH-sensitive nanoniosome containing hedera helix extract with size of 75.1 nm, PDI of 0.351, zeta potential of -20.6 mV, encapsulation rate of 80.1±2.2 and a maximum drug release rate of 57.2% after 72 hr. We also compared the antibacterial activity of the extract in the encapsulated form with the free form and the results showed that the nanosystem containing HH extract has more antibacterial activity. The interaction of the HH extract with the niosomes was also investigated and it was found that nanosystems and the extract have no interaction with each other. Fluorescent imaging also confirmed the successful entry of niosomes into cancer cells, and finally it was found that the synthesized Nio-HHE, in addition to increasing the effect of the HH extract on breast cancer cells BT-474 and MCF-7, can reduce side effects on normal cells. Our study also proved that HH extract had no cytotoxic effects on normal cells which indicated it could be used as a safe anti-cancer agent.

CONCLUSION

In this study, for the first time, we succeeded in introducing a novel method based on phytotherapy and nanotechnology to be used in the treatment of breast cancer by extracting the Hedera helix plant and encapsulating the extract within the niosomal formulation. The nanoparticles synthesized in this study, in addition to having appropriate physicochemical properties, were also sensitive to temperature and pH, which increase the release of drug in response to this stimulus is one of the advantages of this study, which can overcome to the lack of differentiation between cancer and normal cells which is one of the biggest problems in chemotherapy. Also, by adding phospholipids to niosomal formulations, in addition to vanquishing the instability of niosomes, which is considered as problems in applying them, we were able to increase the encapsulation efficiency of synthesized niosomes and facilitated their uptake by cancer cells and in all, improve their function. Successful results of our study showed that niosomes containing Hedera helix extract with proper size, morphology, surface charge and drug release pattern, can not only improve the antibacterial activity of Hedera helix extract compared to free form of it but also can improve its therapeutic effects on breast cancer cells and reduce its adverse side effects on normal cells. Although the results of this study confirm the successful use of synthesized nanoniosomes in breast cancer treatment, it has a long way to go before its clinical use and requires further research.

ACKNOWLEDGMENTS

This project was funded by a grant from Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

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