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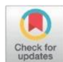

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Original Article

Anticancer Properties of Nanoniosomes Containing Ethanolic and Methanolic Extracts of *Citrus Limon* Peel on SKBR-3 Breast Cancer Cells

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Abstract

Introduction: The adverse effects of chemotherapy in treating breast cancer have prompted research into identifying and using herbal anticancer compounds with suitable carriers. Accordingly, in the current study, we intended to investigate the effects of niosome-formulated methanolic (Nio/ME) and ethanolic (Nio/EE) extracts of *Citrus limon* peel on SKBR-3 breast cancer cells.

Materials and Methods: The SKBR-3 breast cancer cell line was subjected to niosomal formulation of ethanolic and methanolic extracts of lemon peel and doxorubicin. The effects on cytotoxicity, cell death, and migration via MTT, wound healing assay, and flow cytometry analysis were investigated.

Results: MTT observations demonstrated that 200 µg/ml of extracts and 0.5 µM doxorubicin were appropriate for loading in the niosome and administered to the cells for 48 hours. In flow cytometry, the apoptosis rate significantly increased for ethanolic and methanolic extract formulations compared to pure extracts. However, encapsulated doxorubicin had a milder toxicity than doxorubicin alone ($p \leq 0.05$). Wound healing assay demonstrated significant anti-migratory effects of encapsulated extracts. The extracts had a significant synergistic cytotoxic effect with the drug.

Conclusions: Lemon peel extract has anticancer properties similar to doxorubicin. Additionally, the niosomal formulation demonstrated the ability to load and release the extracts and drug efficiently to the breast cancer cell.

Keywords: Nanoniosome, Cancer, Herbal, Apoptosis, Cytotoxicity, Scratch Assay

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Introduction

Breast cancer is one of the most common malignant diseases and the second leading cause of cancer death in women.[1] In addition to the high prevalence of breast cancer in Iran, Iranian women suffer from this disease a decade earlier than the usual age in developed countries. Despite treatment following surgery, many patients experience recurrent metastatic cancer.[2] In *in vitro* studies on breast cancer cells, three cell types are usually used, which differ in terms of estrogen receptor (ER), progesterone receptor (PR), and HER2. The SKBR-3 cell line is negative for ER and PR receptors but positive for HER2.[3] Treatment options for breast cancer include surgery, radiotherapy, and chemotherapy. However, due to the young age of the patients, there is a need for novel therapeutic approaches, such as integrating nanotechnology into the field of medicine.

Citrus fruits, including lemons, are the main source of flavonoids and have long been assumed to have protective properties against cancer. Citrus peel is a waste (byproduct) of citrus juice extraction and consists of three layers: flavedo, albedo, and endocarp.[1] Lemon peel makes up about 13% of the whole fruit and 65% of its waste. Flavonoids have antioxidant properties against free radicals. In addition, there are several reports that flavonoids can act in different stages of cancer.[2] A large part of the biologically valuable compounds of this plant are found in its waste. The peels of citrus fruits are responsible for the antioxidant, inhibitory, and anti-proliferative activities of flavonoids present in them.[3] The medicinal application of citrus peels can be traced back to the 10th century AD, but the biological activities of specific chemicals in citrus peels have recently been identified.[4,5] Citrus peel has unused potential as a source of medicinal compounds because it contains carotene, essential oils, pectin, and polyphenol compounds.[6] Flavones show anti-proliferative activity against human lung cancer cells (A549), squamous cell cancer (HBT43),[7] gastric cancer, leukemia (HL-60), T-cell leukemia (CCRF) and B16 melanoma cells.[8]

Nano-scale injectable carriers, including niosomes, can

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cross biological barriers, protect the drug, and release an optimal amount of it. Recent advances in nanotechnology have enabled targeted treatment of diseases while reducing drug side effects.[9] Niosomes are non-ionic surfactant vesicles formed by hydrating non-ionic surfactants with or without mixing cholesterol and other fats. Their vesicular system can serve as a carrier for lipophilic and amphiphilic drugs, and their non-ionic nature leads to lower toxicity and slow release, limiting their reaction with cells and enhancing the effect of the encapsulated drug.[10] Niosomes have a substructure consisting of hydrophilic, amphiphilic, and lipophilic components and can accommodate pharmaceutical molecules with a wide range of solubility, such as plant extracts.[11] Based on previous research, we aimed to investigate the effects of niosome-formulated methanolic (Nio/ME) and ethanolic (Nio/EE) extracts of Citrus limon peel on SKBR-3 breast cancer cells.

Materials and Methods

Materials

Human breast cancer cell line (SKBR-3) was purchased from the National Cell Bank of Pasteur Institute (Tehran, Iran). Dulbecco's Modified Eagle Medium (DMEM) and Fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific Co., (Massachusetts, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent was purchased from Melford (UK), and Doxorubicin drug (DOXO) was acquired from EBEWE Pharma (Austria). Sorbitan monostearate (Span 60), Polyoxyethylene sorbitan monostearate (Tween 60), cholesterol, dimethyl sulfoxide (DMSO), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Merck (Germany). RealQ Plus 2× Master Mix Green was from Amplicon (Denmark), and Annexin V apoptosis detection kit FITC, eBioscienceTM was purchased from Thermo Fisher Scientific (USA).

Extraction of Ethanolic and Methanolic Extract of Lemon Peel

In December 2020, *Citrus limon* (L.) Osbeck (Lisbon variety cultivated in the Jahrom region of Fars province, Iran) was collected for experimentation. This variety is characterized by its thick skin and pale yellow flesh. Only intact fruits without any disease were selected and washed with water. Subsequently, the outer layer of the peel, known as the epicarp or flavedo, was carefully removed using a shredder and dried in a shaded area. During this process, efforts were made to avoid getting any mesocarp (albedo) in the peel. 50 g of the dried peel was then pulverized and subjected to extraction using a Soxhlet extractor. The extraction solvent consisted of a mixture of 20% water and 80% ethanol/methanol, and the extraction process lasted for 10 hours. The resulting extract was concentrated using a rotary device, and the samples were subsequently dried in an oven at 40 °C to remove any remaining solvent. The concentrated extract was stored in the refrigerator in the dark until further analysis. To analyze the presence of agricultural pesticide residues, a dried and pulverized lemon peel sample was sent to Marjaan Khatam Co. in Tehran, Iran.

Radical Scavenging Activity (RSA Measurement)

The RSA test was employed to assess the antioxidant properties of the extracts. Ascorbic acid served as the standard for comparison. Concentrations of 3, 6.25, 12.5, 25, 50, 100, 200, 300, and 400 mg/ml were prepared from both the samples and the standard. Each sample and standard was replicated six times in a volume of 50 µl and added to all six wells. Additionally, the first three replicates received 150 µl of DPPH reagent (2 mg in 50 ml of methanol), while the second three replicates were treated with 150 µl of blank methanol as the negative control. After a 30-minute incubation period, the absorbance of the plate was measured at 517 nm using a plate ELISA reader (BioTek Inc., Vermont USA). The percentage of DPPH radical inhibition was subsequently calculated using Equation 1:

Equation 1: $RSA\ (\%) = 100 \times (A0 - A1)/A0$

Where A0 represents the absorption of the negative control, encompassing all factors except the extract, and A1 denotes the sample absorption. Standard and sample graphs were constructed based on the resultant absorptions. Subsequently, the slope of each graph was individually plotted, followed by the calculation of the slope of the IC_{50} line by Excel software (i.e., the measure of the antioxidant required for the concentration of DPPH to reach 50% of its initial value).

Synthesis and Loading of Niosomes

Thin film hydration was used to synthesize niosomes as described previously.[9,10] Initially, Span60 and cholesterol were prepared at concentrations of 50 mg/ml, and Tween 60 at 100 mg/ml in chloroform. Then, 0.81 ml of Span 60, 1.35 ml of Tween 60, and 0.70 ml of cholesterol were combined in a 50 ml round-bottom flask. The chloroform was evaporated using a rotary device under vacuum conditions at 60 °C for 60 minutes. The resulting lipid film was hydrated with 5 ml of PBS at 60 °C for 30 minutes. To determine the molarity of the niosomes, the molarity of their constituent components, namely cholesterol, Span, and Tween, per unit volume was taken into account. The overall concentration or molarity of the niosomes was then determined by adding up the concentrations of all their components and calculating the concentration per unit volume (mole/L).

The milky solution containing large multi-lamellar vesicles (MLV) niosomes was sonicated for 10 minutes using an ultrasonic system. Stirring was also used to obtain small unilamellar vesicles (SUV) niosomes. The samples were placed in an ice bath during sonication to prevent

overheating. The size and morphology of the synthesized niosomes were measured by Dynamic Light Scattering (DLS) (Cilas Co., France) and light microscopy. The niosomes were filtered by a 0.45 µm membrane filter to obtain niosomes with a better and more uniform size distribution. The loading method was similar to the blank niosome generation technique with a slight modification. The niosomes were prepared at a final concentration of 200 µg/ml extracts and 0.5 µM DOXO. Specific amounts of Span60, Tween60, and cholesterol were added to a 50 ml round-bottom flask. The chloroform solvent was evaporated using a rotary evaporator at 60 °C at 180 rpm for 120 minutes. After evaporation of the solvent, the thin layer obtained in each separate step was hydrated using 5 ml of a solution containing PBS with ethanolic extract (EE), methanolic extract (ME), or DOXO at 60 °C for 30 minutes. Doxorubicin solution has a red color, while the milky color represents the extract solution. The stained solution that was obtained included the loaded formulations. At this stage, sonication was not performed because it would separate the compound from the niosome.

Determination of Entrapment Yield (EY%)

The entrapment yield of extracts and DOXO was determined by measuring absorbance in a spectrophotometer. The EE, ME, and DOXO had index peaks at wavelengths of 208, 274, and 500 nm, respectively. Calibration curves were recorded to determine the entrapment yield. The loaded formulations of the drug and extracts were centrifuged, and the supernatant solution was analyzed at the mentioned wavelengths to calculate the EY% of extracts and DOXO based on equation 2:

$$\text{Equation 2: } Ey\% = \frac{(\text{Total amount of all} - \text{all in supernatant})\times 100}{\text{The total amount of all}}$$

The remaining pellet was re-dissolved in 5 ml PBS and passed through a 0.45 µm membrane filter to obtain niosomes with a better and more uniform size distribution.[10]

Cell Culture and MTT Assay

The SKBR-3 cell line was cultured in DMEM medium, supplemented with 10% v/v FBS (Invitrogen., USA), penicillin, and streptomycin (AFA CHEMIE., IRAN). The cells were incubated in a humid incubator (Binder Co., Germany) with 5% CO_2 at 37 °C. The culture medium was replaced every two days.[11] In order to assess the cytotoxicity of lemon peel extract, SKBR-3 cells at a concentration of 10×10^3 were cultured in a 96-well plate. The next day, the cells were treated with EE and ME at 25, 50, 100, 200, and 400 µg/ml concentrations. To determine the appropriate dosage of niosomes and achieve the lowest cytotoxicity, niosomes were chosen for initial screening at concentrations of 0.5, 2, 5, and 10 µM. Simultaneously, wells in another column were treated with DOXO at concentrations of 0.5, 1, 2, 4, and 5 µM as the positive control. The treatment was carried out for 24, 48, and 72 hours. The first row and column from the left side were considered blank, and the second column from the left was assigned for untreated control cells. After the incubation period, 5 µl of sterile MTT solution (5 mg/ml) was added to the wells and incubated for 4 hours at a 37 °C incubator. Then, 100 µl DMSO was added, and the plate's absorption was read with an ELISA reader (BioTek Co., USA) at 570 nm. The IC_{50} was calculated with GraphPad Prism 8 software (GraphPad Software, LLC, 2018).

Evaluation of Extract-Drug Combination Effect

Isobologram analysis was utilized to determine the synergistic cytotoxic effects of DOXO and the extracts. SKBR-3 cells were cultured in 96-well plates and treated with combined concentrations of extracts and drug [(0.5 µg/ml/200 mM), (1 µg/ml/100 mM), (2 µg/ml/50 mM), and (4 µg/ml/25 mM), respectively]. The synergistic effect of doxorubicin and extracts on cell viability was investigated in the MTT assay.

Flow Cytometry Analysis

To evaluate the apoptotic impact of extracts, SKBR-3 cells (15×10^5) were exposed to Nio/EE, Nio/ME, and niosomal formulation of DOXO (Nio/DOXO) at a final concentration of 2 µM niosome formulation for 48 hours in six-well plates. Simultaneously, a control group was also cultivated. Following the treatment, the cells were detached with a Trypsin-EDTA solution. Subsequently, the cell suspension underwent centrifugation at a speed of 200×g for 5 minutes, and the cell pellet was washed with PBS. Annexin V and Propidium Iodide (PI) were employed to stain the cells in darkness, as the manufacturer's protocol instructed. After that, analysis of the stained cells was performed using a Cyflow space flow cytometer (Partec Co., Germany). An unstained tube of live cells was included to determine the optimum voltage of the channels.

Additionally, one tube was stained with PI, while cells in another tube were stained solely with Annexin to define the quadrant boundary. The treated cells were stained with both Annexin and PI. Data were analyzed using FlowJo software (FlowJo, LLC, V 10.5.3 2018).[11]

Wound Healing Assay

The assessment of cell migration involved the utilization of a wound healing assay. 500,000 SKBR-3 cells were cultivated in a 24-well plate until they reached 90% confluency. Subsequently, the monolayer of cells was subjected to a scratch in the middle of the well using a yellow pipette tip to create a circle. After removing suspended cells through washing, snapshots were taken simultaneously.[12] In each

well, the niosome formulation of the extracts and the DOXO drug were added at a final concentration of 2 µM. The control samples remained untreated, and their scratched area was considered the control. After 48 hours, the scratched surface was photographed again. The rate of closure for the scratched surface in both the treated and control samples was determined using ImageJ software (version 1.53, NIH, USA). Each treatment was repeated in three wells, and the average was analyzed in GraphPad Prism (version 9, 2020).

Statistical Analysis

The viability rates of extracts and DOXO were determined by calculating their cytotoxicity results, employing a wholly randomized basic design with three replications. A comparison of two or more experimental groups was conducted using the student t-test and two-way ANOVA. The data were then expressed as the mean ± SD. A significance level of $p<0.05$ was deemed to be statistically significant. The half maximal inhibitory concentration (IC_{50}) and the subsequent statistical analysis were determined using GraphPad Prism 9.0 software.

Results

Radical Scavenging Activity of the Extract

The RSA results demonstrate that, at a concentration of 200-400 µg/ml, the extract exhibited the closest resemblance to the standard index regarding scavenging free radicals (Figure 1). Furthermore, the antioxidant activity of the extract exhibited a dose-dependent increase. Statistical analysis was done with Prism 6 software, and a p value of 0.0049 was obtained, which shows that there is a significant difference between the antioxidant property of the sample and vitamin C. The IC_{50} values for ascorbic acid and methanol extract were 22.58 µg/ml and 65.4 µg/ml, respectively. This indicates that the ME demonstrated a lower level of antioxidant activity than ascorbic acid. The analysis of pesticide residues revealed that, out of the 78 pesticides examined, only 0.086 mg/kg of propiconazole was detected in the sample (supplementary data).

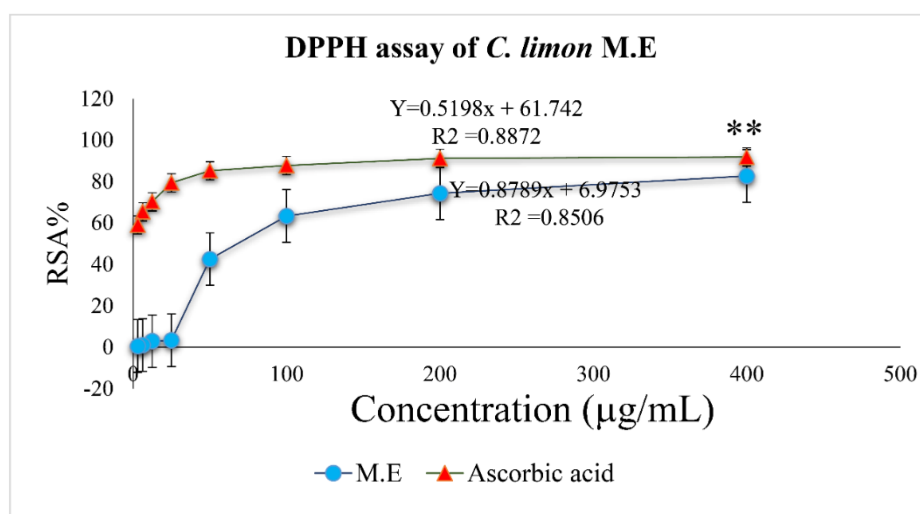


Figure 1. Radical Scavenging Activity of Ascorbic Acid and Methanolic Extract. Data are presented as mean ± SD in 3 replicates. The antioxidant activity of the extract increases in a dose-dependent manner. The results of statistical analysis show that there is a significant difference between the antioxidant properties of the sample and vitamin C (**$p<0.01$).

Analysis of Loaded Niosomes

Dynamic light scattering (DLS) examination was performed on the empty niosome and the niosome loaded with EE, ME, and DOXO, as illustrated in Figure 2. The diameter of individual niosomes was measured to be 69.9 nm. At the same time, the Nio/EE, Nio/ME, and Nio/DOXO exhibited sizes of 347.7 nm, 441.6 nm, and 233.4 nm, respectively. The encapsulation yields (EY) for Nio/EE, Nio/ME, and Nio/DOXO were determined to be 96%, 98%, and 97.41%, respectively. Moreover, Figure 2 presents a light microscope image depicting the unloaded and loaded niosomes, which displayed a spherical morphology without condensation or aggregation.

MTT Assay

The MTT assay was initially conducted to examine the cytotoxic impact of empty niosome, EE, ME, and DOXO (without niosome formulation) over 24, 48, and 72 hours on SKBR-3 cell lines. The aim was to ascertain the suitable concentration of niosome, extracts, and drug for encapsulation in niosome, as well as the appropriate duration of treatment. The findings demonstrated that the administration of DOXO and crude extracts resulted in significant lethality in all cells after 48 hours. The rate of viable cells treated with 2 µM niosomes for SKBR was 85.74%, and higher concentrations of niosome (≥2 µM) led to an increase in cytotoxicity rates (Figure 3A) (*$p<0.05$, **$p<0.01$). Meanwhile, an elevation

in concentrations of M.E and EE led to a decline in viability rate and augmented cytotoxicity (Figure 3B&C) (*p<0.05, ****p<0.0001). Doxorubicin exhibited a lethal impact on cells at a concentration of ≥0.5 μM, as evidenced by the treatment with various doses of DOXO serving as a positive control (Figure 3D) (*p<0.05, **p<0.01, ****p<0.0001). Furthermore, the findings revealed that the cytotoxicity of 200 μg/ml of the extracts was comparable to that of DOXO.

The cytotoxicity rates for SKBR-3 cells treated with EE, ME, and DOXO after 48 hours were 11%, 4%, and 32%, respectively. The IC_{50} values for EE and ME against SKBR-3 were determined to be 200 and 250 μg/ml, respectively. Based on the cytotoxicity assay results on the SKBR-3 cell line, 200 μg/ml for the extracts and 5 μM for DOXO were employed for niosome loading. Ultimately, a concentration of 2 μM was selected for the niosome formulation.

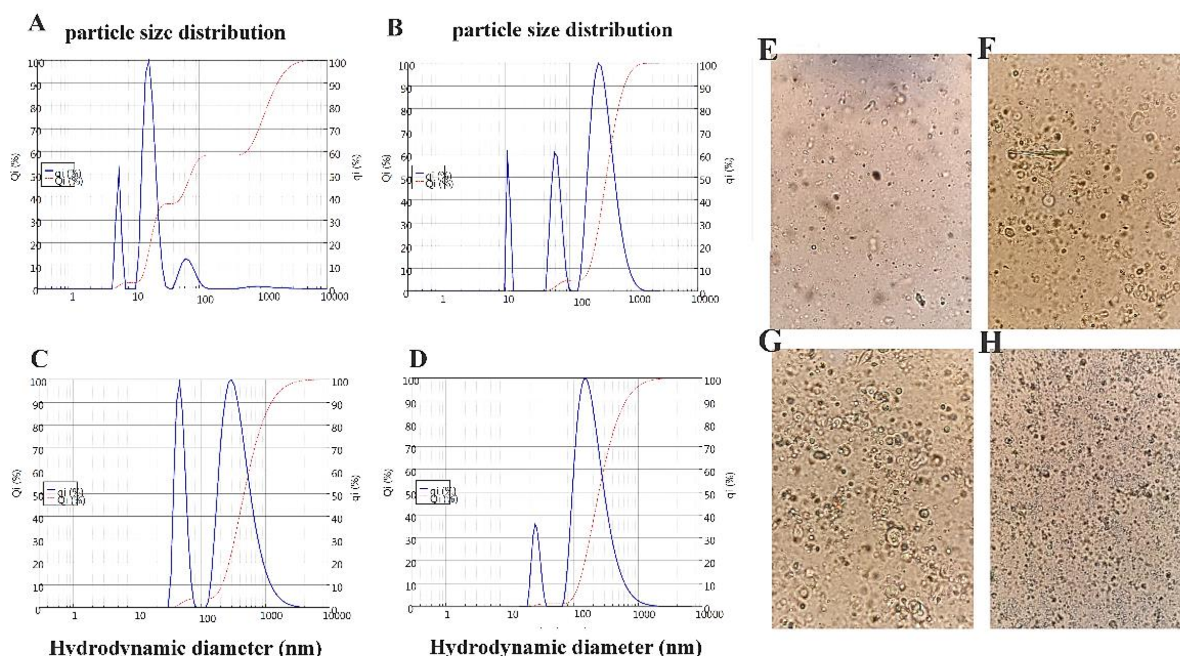


Figure 2. DLS and Morphology Analysis. Left: The size of niosomes (blank Niosome) (A), Nio/EE (B), Nio/ME (C), and Nio/DOXO (D) measured by the DLS method. Right: Light microscope image of blank Niosomes (E), Nio/EE (F), Nio/ME (G), and Nio/DOXO (H). Magnification × 100.

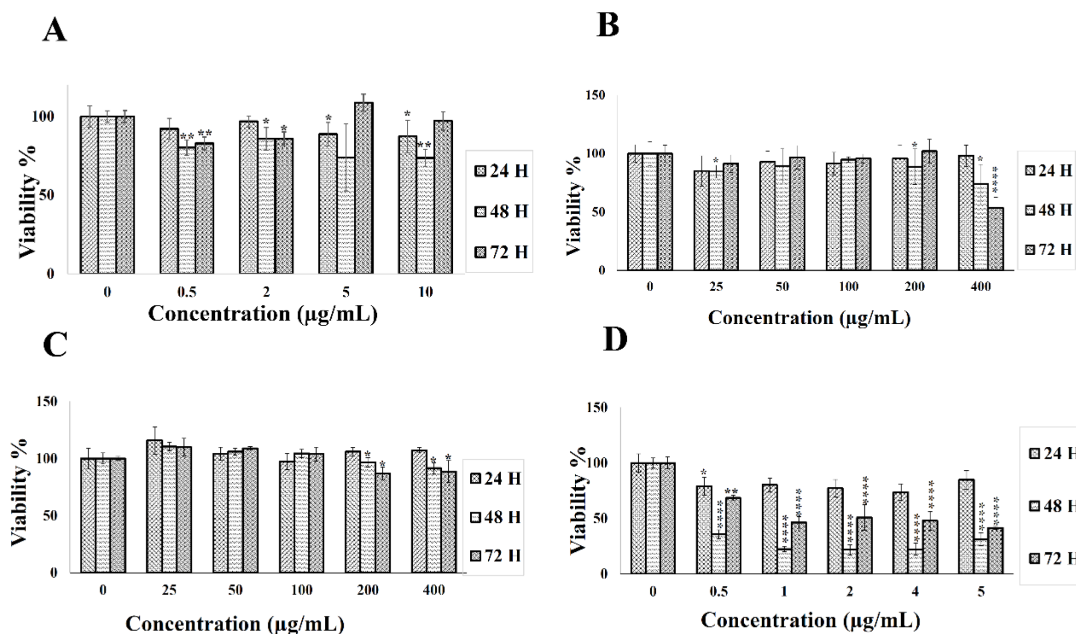


Figure 3. Investigation of the MTT Assay in Groups Treated with A) blank niosome (2, 5, 0.5, and 10 mM concentrations), B) EE, C) ME in (25, 50, 100, 200, and 400 µg/ml concentrations), and D) DOXO (0.5, 1, 2, 4, and 5 µM concentrations) at 24, 48, and 72 hours. Error bars represent the mean ± SD of three replicate experiments. (*p<0.05, **p<0.01, ****p<0.0001)

Synergistic Effects Analysis

The combined effects of the drug and two extracts were evaluated in comparison to separate treatments with each of them through the implementation of the isobologram test during two distinct periods. Following treatment with EE and ME alone for 24 hours, utilizing concentrations of 25, 50, 100, and 200 µg/ml, the average viability rate of SKBR-3 cells was determined to be 91.28% and 99.84%, respectively. For the drug-only treated group, viability was assessed using concentrations of 0.5, 1, 2, 4, and 5 µM, yielding an average viability of SKBR-3 of 77.57%. However, when considering the combined effect of EE and ME with the drug, specifically 0.5/200, 1/100, 2/50, and 4/25, the overall average viability for SKBR-3 decreased to 67.28% and 61.70%, respectively (***p<0.001, **p<0.01, ****p<0.0001) (Figure 4A). In the case of a 48-hour treatment with EE and ME for SKBR-3, the average viability was found to be 89.40% and 106.29%, respectively (****p<0.0001). Following treatment with the drug, the viability was 25.49% for SKBR-3. However, after combining EE and ME with the drug, the viability decreased to 34.26% and 30.74% for SKBR-3 (Figure 4B).

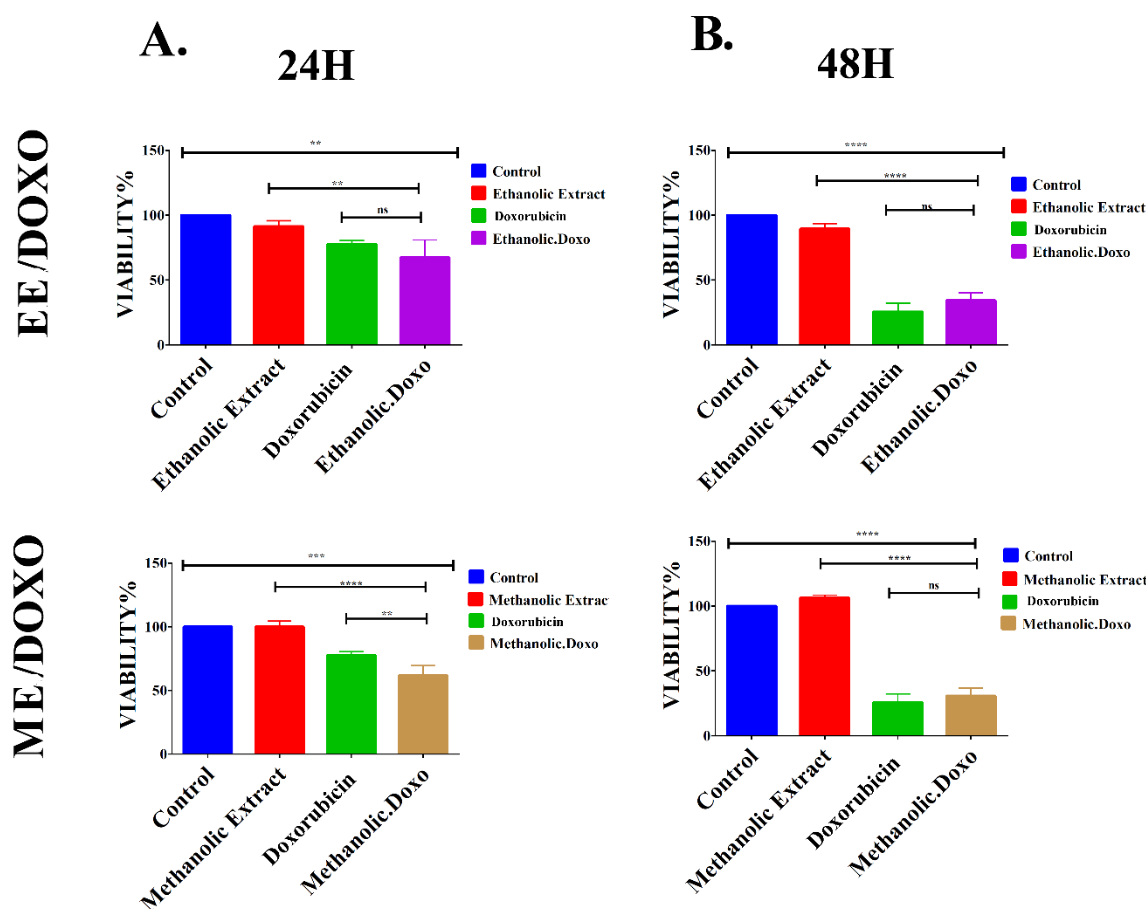


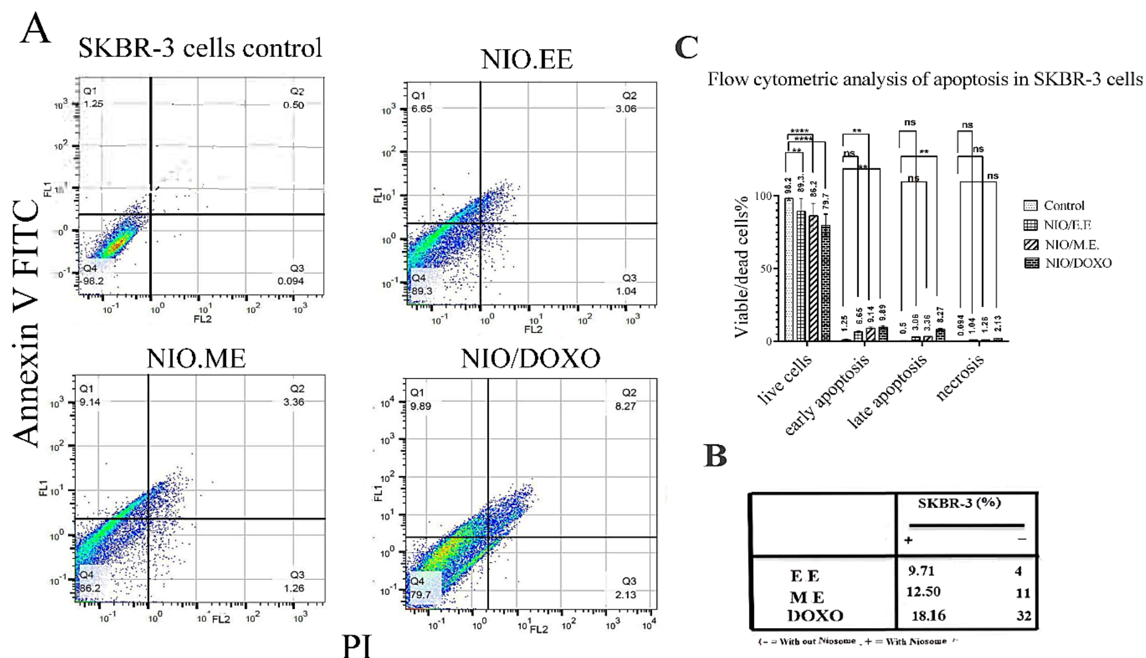
Figure 4. Analysis of the Synergetic Effects of the Drug and Two Extracts on the Viability of SKBR-3 Cells Following 24 and 48 hours. Data were analyzed in Prism 6.0 software and presented as mean ± SD in 3 replicates. A): For 24 hours, the synergistic effects of the extracts and the drug compared to the control and the effect of the extracts alone significantly decreased the viability of SKBR-3 cells. B): The synergistic effects of the extracts and the drug compared to the control and extracts alone showed a significant decrease in cell viability following 48 hours. (**p<0.01, ***p<0.001, ****p<0.0001).

Evaluation of Apoptotic Activity

The apoptotic activity of Nio/EE, Nio/ME, and Nio/DOXO in SKBR-3 demonstrated a significant increase (****p<0.0001) compared to the control group, as depicted in Figure 5A. Notably, early apoptosis in SKBR-3 exhibited a notable rise, with percentages of 6.65% for Nio/EE, 9.14% for Nio/ME, and 9.89% for Nio/DOXO. In contrast, the control group displayed early apoptosis at a rate of 1.25%. Additionally, the late apoptotic increase for SKBR-3 was observed as follows: Nio/EE (3.06%), Nio/ME (3.36%), and Nio/DOXO (8.27%) when compared to the control group (0.5%). Figure 5B compares total apoptotic death with cytotoxicity in the MTT assay for extracts and doxorubicin without niosomal formulation. The statistical analysis of early and late apoptotic death is presented in Figure 5C (**p<0.01, ****p<0.0001).

Migration Assay

The results of the migration assay revealed that, after 48 hours, the control group exhibited only 5.2% of the scratch surface remaining. However, the Nio/EE group showed a significant increase of 200.74%, the NIO/ME group exhibited an 85.28% increase, and the NIO/DOXO group demonstrated a remarkable 237.84% increase in the scratched surface (**p<0.01, ****p<0.0001). These results indicate that the SKBR-3 cells were significantly impacted by the extracts, leading to a notable inhibition of migration in the treatment groups compared to the control group (Figure 6).



	SKBR-3 (%) +	SKBR-3 (%) −
E E	9.71	4
M E	12.50	11
DOXO	18.16	32

(− = With out Niosome , + = With Niosome)

Figure 5. Evaluation of the Apoptotic Activity of Extracts and DOXO after 48 hours of Treatment on SKBR-3 cell lines. A): Early and late-stage apoptotic cells are shown in the graph's upper left and right quadrants, respectively; B) Comparison of toxicity percentage in MTT and flow cytometry in SKBR-3 for extracts and DOXO with and without noisome; C) Statistical analysis of early and late apoptosis occurrence after 48-hour treatment of Nio/EE, Nio/ME, and Nio/DOXO formulations in SKBR-3 cell lines compared to the untreated group. Treatment of SKBR-3 cells with all three niosomal formulations caused a significant increase in early apoptosis (****p<0.0001) and late apoptosis (**p<0.01) compared to the control.

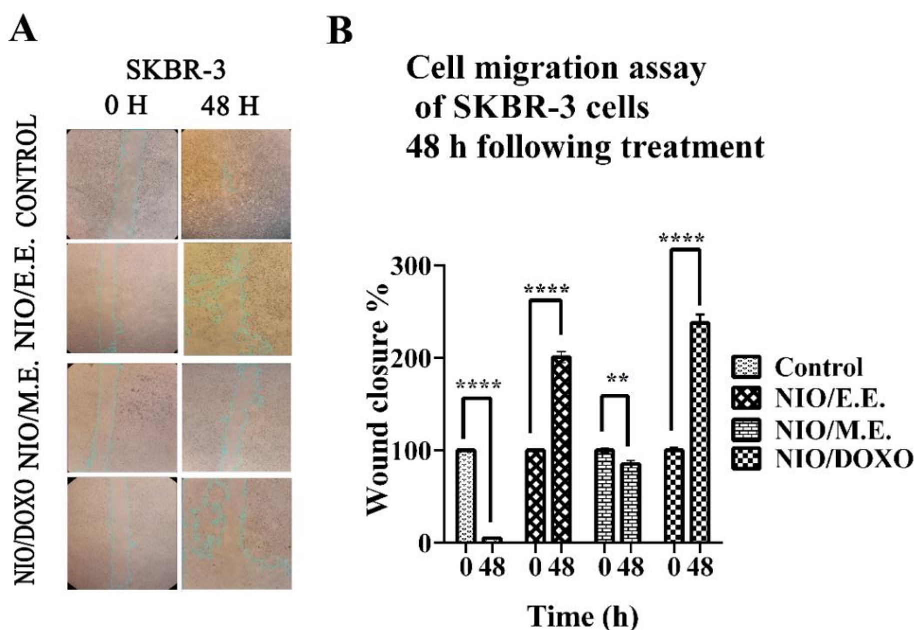


Figure 6. Scratch Test Status with Statistical Analysis 48 hours after Wounding. A) In SKBR-3 cell lines, the percentage of wound area was significantly increased compared to the control, especially in Nio/EE and Nio/DOXO; B). Statistical analysis of Nio/EE, Nio/ME, and Nio/DOXO Effect on SKBR-3 48 hours after wounding (**p<0.01, ****p<0.0001). Error bars represent the mean ± SD of three replicate experiments.

Discussion

The high prevalence of breast cancer and the various side effects of chemotherapy have attracted the attention of scientists towards identifying and developing herbal medicines, as well as utilizing drug delivery methods for controlled release. In 2013, a study examined the relationship between the consumption of citrus fruits in the diet and the risk of breast cancer. The combined results from observational studies indicated an inverse relationship between the two.[11] Another study showed that the flavonoids in the extract could potentially be a factor in preventing breast cancer.[12] Despite their medicinal applications, flavonoid compounds generally have poor solubility in water. As a result, their permeability through the body's cell membrane is low, and they require a carrier.[13] In this research, we utilized a niosomal carrier to load ethanolic and methanolic extracts of lemon peel and doxorubicin. Niosomes have a high capacity to encapsulate hydrophilic, lipophilic, and hydrophobic therapeutic agents, protecting them and providing a more targeted therapeutic effect compared to other oily formulations. Niosomal formulations have shown significant potential in targeting liver cancer tissue.[14] An increase in the retention and preservation of unstable micronutrient elements, including vitamin C, inside the nanoparticles has been reported. The nanoparticles obtained from lemon fruit have high and effective antioxidant activity on human cells.[15] Therefore, this project investigated apoptotic activity, migration inhibition, and synergistic effects of ethanolic and methanolic extracts of lemon peel and drugs on SKBR-3 cells. Simultaneous administration of the extracts and doxorubicin showed a significant decrease in cell viability compared to untreated cells and cells treated with each substance alone. These results confirm the synergistic effect of the drug and the extracts. Similar to our findings, an increase in the antitumor effects of PST001 when combined with fluorouracil has been reported on cancer cells.[16] The size of loaded niosomes in DLS analysis was larger than empty niosomes. The greater dimensions of the filled niosomes, compared to the empty niosomes, indicate the successful encapsulation of the extracts and DOXO within the niosomes. The size of loaded niosomes in DLS analysis was larger than empty niosomes. The greater dimensions of the filled niosomes, compared to the empty niosomes, indicate the successful encapsulation of the extracts and DOXO within the niosomes. The enlargement of niosomes when loading a plant extract is a complex phenomenon that can be influenced by various factors, including the molecular size of the plant extract, the physical properties of the niosomal membrane, the interplay between the components of the plant extract and the niosomal components, as well as the pH, temperature, and loading method employed. Therefore, changes in the size of niosomes might be due to the delicate balance of different factors involved in the formulation formation. In 2017, Mashal et al. investigated the size of niosomes prepared in the absence/presence of lycopene and showed that loading lycopene into niosomes can increase the size of the formulations.[17] The size of niosomes was suitable for avoiding the Enhanced Permeability and Retention Effect (EPR).[18] The entrapment efficiency of ethanol, methanol, and drug nanocarriers was, on average, >95%, indicating successful loading of chemicals into these formulations (*p<0.05). Flow cytometry and MTT analysis showed that the cells treated with niosomes formulation of extracts had a higher rate of apoptotic death. The loaded niosomes increased the anticancer effect of the extracts compared to the unloaded extract. This data indicates the effective function of niosomes for efficient targeted release of extracts to the cells. In a similar study, a reduction in the viability of breast cancer cells after treatment with Carum niosomal extract was reported compared to the extract alone.[19] However, the results for DOXO were just the opposite. Nio/DOXO had a lower death rate than DOXO alone. The slow effect of doxorubicin in the niosome can help prevent the severe and adverse side effects of chemotherapy drugs. This finding demonstrates the effectiveness of niosomes as a drug delivery system for cancer therapy. In a similar study, it has been observed that nanovesicles from lemon extracts inhibit the growth of cancer cells through the TRAIL pathway without affecting normal cells.[20] This observation also confirms the use of niosomes to deliver plant compounds to cancer cells. In the scratch test, the niosome formulation of both the drug and the extracts significantly inhibited cell migration. Park et al. in 2016 also showed that the extract taken from peels of Korean orange could inhibit migration in breast cancer cell lines.[21] In comparing the lethal effects of ethanolic and methanolic extracts with or without formulation, the methanolic extract was slightly more lethal on the SKBR-3 cell line. The biochemical characteristics of the extracted compounds may vary between the ethanolic and methanolic extracts. Therefore, the differences observed in cellular responses to the ethanolic or methanolic extracts can be attributed to different biomolecules dissolved in the two extracts.

Conclusion

The results show that niosomes have a high potential to carry phytochemicals with low solubility. The niosomes containing lemon peel extracts significantly induced apoptosis cell death in breast cancer cell SKBR-3 and inhibited its migration in a targeted manner. Hence, incorporating phytochemicals and conventional chemotherapeutic agents (e.g., doxorubicin) within niosomes augments the efficacy of anticancer endeavors. Evaluating the phytochemical constituents present in lime peel, elucidating the intracellular pathways and targets of these constituents, and conducting *in vivo* investigations using animal models can elucidate the anticancer properties of this valuable species.

Authors' Contributions

All authors contributed appropriately to this study.

Conflict of Interest Disclosures

This work was extracted from the Ph.D. thesis of Toktam Deylami. The authors declare that they have no conflicts of interest.

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