Delivery of Melittin Loaded Niosomes for Breast Cancer Treatment: an in-vitro and in-vivo Evaluation of Anti-cancer Effect

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Research

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Abstract

Background

Melittin, a peptide component of honey bee venom, is an appealing candidate for cancer therapy. In the current study, Melittin and Melittin loaded niosome had been optimized and assessed the anticancer effect an In-Vitro on 4T1 and SKBR3 breast cell lines and In-Vivo on BALB/C inbred mice. "Thin-layer hydration method" was used for preparing the Niosomes; different niosomal formulations of Melittin were prepared and characterized in terms of morphology, size, Polydispersity index, encapsulation efficiency, release kinetics, and stability. A niosome was formulated and loaded with Melittin as a promising drug carrier system for chemotherapy of the breast cancer cells. Hemolysis, apoptosis, cell cytotoxicity, invasion and migration of selected concentrations of Melittin, and Melittin loaded niosome were evaluated on 4T1 and SKBR3 cells using hemolytic activity assay, flow cytometry, MTT assay, soft agar colony assay, and wound healing assay. Real-time PCR was used to determine the genes expression. 35 BALB/c inbred mice were used; then, the histopathology, *P53* immunohistochemical assay and estimate of renal and liver enzyme activity for all groups had been done.

Results: This study showed Melittin loaded niosome is an excellent substitute in breast cancer treatment due to enhanced targeting, encapsulation efficiency, PDI, and release rate and shows a high anticancer effect on cell lines. The Melittin loaded niosome affect the genes expression by studied cells were higher than other samples; down-regulates the expression of *Bcl2*, *MMP2*, and *MMP9* genes while they up-regulate the expression of *Bax*, *Caspase3* and *Caspase9* genes. Also enhanced the apoptosis rate and inhibited cell migration, invasion in both cell lines compared to the Melittin samples. Results of histopathology showed reduce mitosis index, invasion and pleomorphism in Melittin loaded niosome. Renal and hepatic biomarker activity did not show a significant difference in Melittin loaded niosome and Melittin compared to healthy control. In immunohistochemistry, *P53* expression did not show a significant change in all groups.

Conclusions: Our study successfully declares that Melittin loaded niosome had more anti-cancer effects than free Melittin. This project has demonstrated that Niosomes are suitable vesicle carriers for Melittin, compare to free form.

Background

These days Breast cancer increased in the female population [1]. Therapeutic options for the treatment of breast cancer are dependent on the specific biological characteristics of the tumor. If the tumor is low grade, node-negative and estrogen-receptor-positive, hormone therapy may be recommended, however, if the tumor is of high grade and node-positive, chemotherapy is generally practiced before targeted therapies depending on the hormonal/ErBb2 status of the tumor [2]. Anthracyclines, such as doxorubicin, Epirubicin and taxanes are the current therapeutics for breast cancer treatment [3]. Nevertheless, fighting with breast cancer still faces main obstacles, and finding new agents with the potential to be used in combination with current medicines to improve the therapeutic outcome and reduce the side effects is highly appreciated.

The two main components of honey bee venom are phospholipase A2 and Melittin. Melittin has a pro-apoptotic effect and shows anti-tumor activity [4]. Melittin (C131H229N39O31) is an amphiphilic peptide with 26 amino acid residues [5] and has membrane-perturbing effects, like pore formation, fusion, and vesiculation [6, 7]. Melittin has been used in tumor-bearing mice research because of its cytotoxicity on different tumor cell lines and its capacity to inhibit cell growth or induce necrosis and apoptosis [8, 9]. Melittin can induce apoptosis, cell cycle arrest, growth inhibition in different cancer cells [8, 10-12] and immunoregulatory activity [13]. Honey bee venom can enhance T lymphocyte esterase expression in sarcoma mouse and increase T lymphocyte functions [14]. Melittin augments Th1 cells function and is used for the therapy of low immune function, cancer and viral infection [15].

The 4T1 is a transplantable breast tumor cell line that has highly tumorigenic, invasive and, unlike most tumor models, so can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant areas such as lymph nodes, liver, blood, brain, lung, and bone [16]. 4T1 tumor cells are easily transplanted into the mammary gland so that the primary tumor grows in the correct area [16]. As breast cancer in human, 4T1 metastatic disease develops spontaneously from the primary tumor also, the progressive spread of 4T1 metastases to the draining lymph nodes and other important organs is similar to that of human breast cancer [16].

The *Bcl2* protein family, of which *Bax* is a member, plays a critical role in cell death or survival [17, 18]. *Bcl2* is expressed in solid tumors, such as breast, colorectal, prostate, stomach, lung, ovarian, and cancers [18]. *Bcl2* family proteins expressed in normal mammary tissue [18]. *Bcl2*-positive expression in breast cancer a sign of estrogen receptor functional activity [19, 20]. The balance between *Bax* as a pro-apoptotic and *Bcl2* as a anti-apoptotic protein levels is necessary for the regulation of apoptosis and overexpression of *Bax* leads to apoptosis in cells, suggesting that tight regulation of *Bax*, from transcription to post a translation, is important for cell survival [21] .

MMP2 and *MMP9* are mainly secreted by tumor cells and stromal cells in the form of zymogens, they play main role in degrading extracellular matrices and metastasis and promoting tumor invasion [22, 23]. Melittin has anticancer effects on 4T1 breast cancer cells with up-regulation on *Bax*, *Mfn1*, *Caspase3* and *Caspase9* and down-regulation on *Bcl2*, *Drp1*, *MMP2* and *MMP9* genes so it can be a best candidate for further research on breast cancer treatment [24]. Therefore, combination therapy with more precise technique to maximize the efficacy of the therapy is valuable. Nanoparticles conjugation with chemotherapeutic drugs and natural compounds with anti-cancer effect showed some promising outcome, with many of them approved for treatment of different cancer types [25].

Nanotechnology is an innovative scientific field that takes account of the eccentric features at the nanoscale. Nanoparticles provide a high surface area to mass ratio and usually interact efficiently with their surroundings, but they can work as contained carriers for their constituent molecules rather than the same molecules in solution [26]. Therefore, nanoparticles are promising carriers for targeted delivery of therapeutic substitutes. Nanoparticles have been designed for optimizing size and characteristics to magnify the biodistribution of cancer drugs in the bloodstream. They can transfer their loaded active drug to cancer cells by selectively using tumors' specific stimuli [27, 28]. Drug resistance is another obstacle that hinders the efficacy of molecularly targeted and precise chemotherapeutic operators and can reduce nanoparticle applications. Multifunctional and multiplex nanoparticles are now being actively investigated as aiding, personalized, and tailored cancer medication. Drug delivery systems are characterized as formulations aiming to convey a drug to the desired area of action through the body [29]. Niosomes are special drug carriers developed by the self-association of cholesterol and nonionic surfactants in an aqueous phase [27, 29]. They are an alternative to phospholipid vesicles to encapsulate hydrophobic and hydrophilic drugs providing sufficient encapsulation capability, biocompatibility, biodegradation, low preparation cost, and ample stability [28, 29].

The presented study aims for a more precise and effective drug as a cancer remedy with approaching combination treatment, using Melittin and loaded into Nanoniosomes. In this work, the impact of niosomal formulations had been assessed on 4T1 and SKBR3 breast cancer cell line proliferation with free drugs and evaluated its possible effects on *Bax*, *Bcl2*, *Caspase3*, *Caspase9*, *MMP2* and *MMP9* mRNA expression and effect on inbred BALB/c mice.

Result & Discussion

3.1 Melittin-loaded Niosomes Characteristics:

The encapsulation efficiency (EE) and the size of the niosome much depend on the type of surfactants and the volume of cholesterol (i.e., lipid) in the niosomal structure because any change in the chemical species and chemical formation undeviatingly affects the hydrophilic-lipophilic balance (HLB) in the niosomal formulation. A suitable drug delivery system must have high encapsulation efficiency, a small size, and a structure to carry an ample amount of drug, penetrate the target tissue, and release the drug molecules inside the target tissue. However, there is an obscure association between the size and encapsulation efficiency in the niosomal formulations. Various formulations have been prepared and investigated to optimize the arrangement. **Table 1** shows the size, polydispersity index (PDI), and encapsulation efficiency (EE) of the niosomal formulation in terms of the span60 percentage (i.e., in the Span 60/Tween 60 mixture) and cholesterol content for a specific amount (1mg) of Melittin as a drug molecule, then sonicated for five minutes to provide more uniform Niosomes. Melittin has multiple hydrophilic groups (e.g., OH, and NH), which inaugurates an interaction between Melittin and the hydrophilic chain of niosome. Hence, a unification of two surfactants with low and high hydrophilic-hydrophobic balance (HLB) may result in Melittin's tremendous encapsulation with the small size of niosomal formulation. Tween 60 is a nonionic surfactant with a high hydrophilic moiety, while Span 60 is a nonionic surfactant with a hydrophobic fraction.

Consequently, varying Span 60/Tween 60 ratios could accurately regulate the hydrophilic-lipophilic balance (HLB) of the surfactants and directly modify their cooperation with drug molecules [32]. It can be concluded from **Table 1**, adding tween60 to the formulation represents the zenith encapsulation efficacy and PDI, while it provides the smallest size (Table1, T2, and T5). As shown in **Table 1**, T1 to T3, when the surfactant/cholesterol ratio was 1:1, the particles' mean size is smaller than when the ratio changed to 2:1, due to a thicker lipid layer, which cholesterol provided. However, applying tween60 to the formulation provided an adequate EE and size; utilizing it separately in cholesterol combination without span60 resulted in a larger size and less EE (Table1, T3, and T6). It was evident from the experiment results that span60 cannot provide either a suitable encapsulation efficacy and particle size (Table2, T1, and T5). All the results conclude that the combination of span60 and tween60 with the cholesterol possesses the optimum drug carrier for hydrophilic drugs like Melittin [33, 34].

3.2 Morphological characterization:

The morphology of Melittin-loaded Niosomes was assessed using SEM and TEM methods. In this study, the Niosomes particle size was less than 50nm, much smaller than DLS observation. This variance can be due to the difference between SEM and DLS techniques [28]; in SEM, the dried samples are examined, but in DLS, the samples might be hydrated; thus, the particles' size in the DLS test is more prominent. The examination demonstrates a smooth surface, spherical form, and separated firm boundaries with a uniform distribution. The size of nanoparticles has been assessed using Dynamic Light Scattering DLS. As **Figure 1** shows, the average diameter is 121.4nm, which represents the optimum formulation size. As it had been mentioned before, other formulations did not provide acceptable sizes for drug delivery applications.

3.3 In-Vitro drug kinetic and release studies of Melittin from Niosomes:

To investigate the In-Vitro drug release, every selected formulation drug release profile was examined for 72 hours in 7.4, 6.5, and 5.4 pH at the body temperature. As it can be seen in the "Release" diagram (**Figure 2**), the free drug first had burst in the bloodstream (82.19% during first 8 hours); after 24hours, it had reached to monotonous release manner for the next hours. Niosomal Melittin release profile surveillances showed that in 7.4 pH, 43.45% of the drug had penetrated in the first eight hours; this rate increased to 44.91 in 6.5 pH and 51.29% in 5.4 pH. After 72hours, 72.19%, 80.81%, and 92.11% of the drug released into the bloodstream in 5.4pH, 6.5pH, and 7.4pH, respectively, attributed to the acidic condition Niosomes-swelling structure [35]. Niosomes' acidic departure is related to electrophilic addition reactions. The drug-loaded Niosomes had studied for the release rate. Acidic pH crushed the Niosomes structure, increasing the release rate, increasing the toxicity as the tumor wards habitually have the acidic condition [36]. Also, the acidic condition affects the Melittin and increases the osmotic pressure, which induces more cytotoxicity [37, 38]. Melittin's release data had been mathematically measured, in zero-order, first-order, Korsmeyer-Peppas, and Higuchi's orders, in three pH range (7.4, 6.5, and 5.4) for 72 hours in body temperature, **Table 3**. Free drug release followed the first-order model, with the rate of $R^2=0.9643$, representing a drug-concentration release (this applies for Melittin, as a separate free drug). Melittin-loaded Niosomes had followed the Korsmeyer-Peppas model (n) in either 7.4, 6.5, and 5.4 pH. This fact declared that the release mechanism is the diffusion-erosion arrangement. The release rate in each, 5.4, 6.5, and 7.4 pH, are $R^2=0.9431$ with $n=0.4021$, $R^2=0.9297$ with $n=0.4141$, and $R^2=0.9003$ with $n=0.4454$ respectively [39].

3.4 Physical Stability Study of Niosomal Melittin:

Vesicle size, polydispersity index (PDI), and encapsulation efficiency (EE) were analyzed by putting them at 4°C and 25°C, and on days 0, 14, 30, and 60 after the preparation to pick the optimal Melittin Niosomal formulations and physical stability. Interestingly, the observations demonstrated that the temperature affected neither the size of particles, PDI, or EE percentage and possesses the minimum size with the mean of 121.4nm, maximum PDI (0.211), and EE (79.32%) on the day the formulation just prepared. As shown in the stability figure (**Figure 3**), in the following days to day60, the temperature affected all the parameters. Increasing the temperature caused size expansion, more PDI, and EE reduction. The EE reduction is due to the rise of drug release in terms of temperature increase [34]. As the temperature can affect the rigidity and elasticity, growing the pores of the Niosomes; could be effective on the particles size and PDI and increase either of them and reduce the EE to its minimum amount (55.19%). As can be concluded from the stability results, the stability is better at 4°C attributed to the rigidity and elasticity of the niosomes, because at 25°C, the grown pores caused bigger size, more PDI, and less EE.

3.1. Hemolytic Activity of Melittin in BALB/c Mice Erythrocytes

A powerful hemolytic activity was observed in the purified Melittin from honey bee venom. The findings indicated that Melittin had a powerful hemolytic activity at the concentrations of above 0.5 µg/ml (**Figure 4**). 0.125 µg/ml and 0.25 µg/ml concentrations of Melittin did not indicate considerable lysis effect. The HD_{50} value obtained 2 µg/ml indicating is the concentration, at which Melittin shows 50% of the hemolytic activity of the positive control.

Melittin is composed amino acid residues and Melittin's hemolytic activity has been demonstrated and It is known to have a powerful hemolytic activity against mammalian and bacterial cells due to its weak cell specificity [40, 41].

3.5 Cell proliferation assay

The treatment of two breast cancer cells (4T1 and SKBR3 cells) with niosomal formulation resulted in a higher inhibitory effect (less cell viability) compared to free drug solutions. To determine the inhibitory effect of individual Melittin as a free form and Melittin loaded niosome as a niosomal form on 4T1 and SKBR3 cells. A dose-response experiment had been performed for both groups. As indicated in individual treatments with the free form and the niosomal form resulted in growth inhibition of 4T1 and SKBR3 cells in a dose-dependent pattern.

IC50 value was evaluated in all study groups on 4T1 mice breast cancer cell line after 48 and 72 h treatment. In study groups (Melittin and Melittin loaded niosome) IC50 values respectively were 143.20 µg/ml and 75.58 µg/ml after 48 h, and 40.62 µg/ml, 27.17 µg/ml after 72h. According to the results in all groups, IC50 remarkably decreased after 72 h compared to 48 h ($P<0.001$ ***) (**Figure 5a**). In SKBR3 human breast cancer cell line after 48 and 72 h treatment IC50 value was evaluated. In Melittin and Melittin loaded niosome groups IC50 values respectively were 87.87 µg/ml and 47.65 µg/ml after 48 h and 50.56 µg/ml, 31.05 µg/ml after 72h treatment. According to the results in study groups IC50 remarkably decreased after 72 h compared to 48 h ($P<0.001$ ***) (**Figure 5b**). The 4T1 cell line after 48 h of treatment through Melittin loaded niosome (IC50: 75.58 µg/ml) compared to Melittin (IC50: 143.20 µg/ml) IC50 was decrease ($P<0.001$ ***). After 72 h of treatment, the results showed the Melittin loaded niosome (IC50: 27.17 µg/ml) compared to Melittin (IC50: 40.62 µg/ml) was decrease ($P<0.001$ ***) (**Figure 5c**). The SKBR3 cell line after 48 h of treatment, the results showed the Melittin loaded niosome (IC50: 47.65 µg/ml) compared to Melittin (IC50: 87.87 µg/ml) was decrease ($P<0.001$ ***). After 72 h of treatment, the results showed the Melittin loaded niosome (IC50: 31.05 µg/ml) compared to Melittin (IC50: 50.56 µg/ml) was decrease ($P<0.001$ ***) (**Figure 5d**).

The (**Figure 6**) shows the Niosome and Melittin loaded niosome effects on the MCF10A cell line. The MTT assay was performed for evaluation of cytotoxic effects of different Niosome dilution on MCF10A cells. Niosome dilutions has no significant cytotoxic effects on MCF10A cells separately (**Figure 6a**). As the Melittin loaded niosome concentration increased, cell viability decreased. So, the 64 and 128 µg/ml compared to control cell viability was decreased ($P<0.01$ ** and $P<0.001$ ***) (**Figure 6b**). These results indicated that Melittin in free and niosomal forms had more cytotoxicity effect on 4T1 cells and as a model for breast mice mammary epithelial cancer cells compared to SKBR3 cell line after 72h of treatment. The IC50 concentrations were then utilized to generate fixed ratios for subsequent combination experiments and the calculation of combination index (CI).

Melittin exemplifies this large class of membrane-active peptides that manifest membrane-disrupting activity when incorporated into traditional bilayer delivery systems (i.e., liposomes) [42, 43]. Melittin's action is a physical and chemical disruption of membrane structure resulting in profound compromise of the cell permeability barrier by lysis [43-46]. The peptide partitions into the cell membranes as a monomer, followed by oligomerization into toroidal or barrel stave structures that facilitate pore formation to effect cell death [9, 47]. Melittin have important anti-cancer effects on various types of cancers, including breast cancer so nanocarrier allows accumulation of Melittin in murine tumors In-vivo and a dramatic reduction in tumor growth without any apparent signs of toxicity since nanocarriers selectively delivered Melittin to multiple tumor targets, including cancer cells [9, 47]. In another study the synergistic co-delivery of doxorubicin and Melittin using f nanoparticles for cancer treatment in LC-MS/MS indicated that the co-delivery system of Doxorubicin-Melittin loaded CA-MNPs is highly capable to be used in magnetically targeted cancer therapy [48]. Melittin nano-liposomes would have a better application in hepatocellular carcinoma cells (HCC) therapy due to induced apoptosis in hepatic carcinoma cells In-Vitro and vivo and inhibit hepatocellular carcinoma in LM-3 xenograft tumor model [49].

3.6 Wound healing assay

Migration is one of the important characteristics of cancer cells and promotes cancer metastasis. To determine the effect of Melittin and Melittin loaded niosome on migration and invasion, *In-Vitro* wound (scratch) assays were performed in 4T1 and SKBR3 cells and the wound healing rate was monitored through the complete closure of the scratched. As shown in (**Figure 7**), cell migration on 4T1 and SKBR3 cell lines that treatment with Melittin loaded niosome was lower than Melittin ($P<0.001^{***}$). 4T1 and SKBR3 cells were investigated using wound healing assay 72 h.

Migration of SKBR3 cell line was decreased by treatment with Melittin loaded niosome with an increase on scratch width (μm) compared to Melittin ($P<0.001^{***}$) (**Figure 7a**). 4T1 cell migration was decreased by treatment with Melittin loaded niosome and increase on scratch width (μm) compared to Melittin ($P<0.001^{***}$) (**Figure 7b**).

A wound-healing migration assay was showed and demonstrated that Melittin inhibited the vascular endothelial growth factor migration of non-small cell lung cancer cells, when compared with the control group [50]. The development of most human cancers, primary cells move out and invade the neighboring tissues and ultimately travel to distant sites to make new colonies, so targeting cancer cell migration and invasion is an important and necessary aspect of cancer chemotherapy [51, 52].

Soft agar colony assay

To further demonstrate the inhibitory effect of Melittin and Melittin loaded niosome on cancer cells growth, we performed the soft agar colony formation assay. The number of colonies was concentration-dependently decreased by Melittin and Melittin loaded niosome compared to control (**Figure 8**). Number of colonies in SKBR3 cell line was decreased by treatment with Melittin loaded niosome compared to Melittin ($P<0.001^{***}$) (**Figure 8a**) and in 4T1 cell line the results was same ($P<0.001^{***}$) (**Figure 8b**).

3.3. Flow cytometric analysis

Apoptosis of breast cancer cells was measured by double staining using annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI). In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is exposed to the external cellular environment due to translocation from the inner to the outer surface of the plasma membrane. The flow cytometric analysis diagram of Melittin and Melittin loaded niosome provided in SKBR3 (**Figure 9a**) and 4T1 breast cancer cell lines (**Figure 9b**).

To further determine apoptosis induction of 4T1 and SKBR3 cells were stained with Annexin-V/PI assay, followed by flow cytometry, and were compared with cells treated with Melittin and Melittin loaded niosome. The flow cytometric analysis indicated a significant induction in the percentage of apoptosis rate in both groups of cells treated with IC₅₀ of Melittin and Melittin loaded niosome.

Annexin V could be served as a sensitive probe for the flow cytometric analysis of cells undergoing apoptosis due to its high affinity for PS, even when it conjugated with FITC. Furthermore, PI can stain the DNAs in the flow cytometry due to its intercalating property. PI stains the dead cells as it cannot penetrate the membrane of live cells and apoptotic cells. Therefore, it is useful to differentiate necrotic, apoptotic, healthy, and dead cells.

The results demonstrated that the simultaneous administration total apoptosis of Melittin (%), and Melittin loaded niosome (%) in SKBR3 cell line (**Figure 9a**) and Melittin (%), and Melittin loaded niosome (%) in SKBR3 cell line (**Figure 9b**) enhances the total apoptosis in both studied cancer cells. These results are in agreement with the cytotoxicity data obtained by MTT assay (**Figure 6 a&b**).

Breast cancer cell invasion and growth of tumor cells can be inhibited by Melittin [10]. Combination therapy with anticancer drugs has is a well-known approach in cancer treatment. Plant-derived drugs are desired for anticancer treatment as they are secure, accessible and also when are combined with anti-cancer agents, they are able to exert synergistic therapeutic effect, decrease the doses, toxicity and drug resistance of chemotherapeutic agent [53]. Hesperidin [54, 55], Piperine [56], BV and Melittin [57, 58] have also shown anti-cancer activities on breast cancer cells.

3.7 Gene expression analysis by Real-Time PCR

The inhibitory effect of drugs might be due to the regulation of the expression level of different genes inside the cells. It has been reported that the Melittin and Melittin loaded niosome could affect the expression level of different genes inside the breast cancer

cells. Therefore, the expression of eight different genes (*Bax*, *Bcl2*, *Caspase3*, *Caspase9*, *MMP2* and *MMP9*) inside the two breast cancer cells (4T1 and SKBR3) were measured after treatment of these two cancer cells with different samples containing drug molecules. (**Figure 10a**) shows the expression levels of *Caspase3* gene in SKBR3 cell line treated by Melittin and Melittin loaded niosome. According to the figure, Melittin loaded niosome had higher expression level of *Caspase3* than the Melittin group ($P<0.001^{***}$). (**Figure 10b**) shows the expression levels of *Caspase3* gene in SKBR3 cell line that treated Melittin and Melittin loaded niosome. *Caspase9* expression levels in group with Melittin loaded niosome treatment was higher than the Melittin group ($P<0.001^{***}$). However, in (**Figure 10c**) Melittin loaded niosome has more *Bax* expression levels in cells than Melittin ($P<0.001^{***}$). According to *Bcl2* gene expression levels in the SKBR3 cell line that treated by Melittin loaded niosome lower expression level than the Melittin ($P<0.01^{**}$) (**Figure 10d**).

On the other hand, the expression levels of *Caspase3* (**Figure 11a**) and *Caspase9* (**Figure 11b**) genes in 4T1 cell line treated by Melittin loaded niosome was higher than Melittin ($P<0.001^{***}$). Also indicate the mRNA levels of *Bax* in 4T1 cells treated by Melittin loaded niosome was increased compare to Melittin ($P<0.001^{***}$) (**Figure 11c**). The results revealed that Melittin loaded niosome increase *Bcl2* expression levels in 4T1 cells compared to Melittin ($P<0.001^{***}$) (**Figure 11d**). As shown in figures related to 4T1, the increased *MMP2* and *MMP9* gene expressions levels can be seen in Melittin loaded niosome comparison with the Melittin ($P<0.01^{***}$) (**Figure 11e & 11f**).

According to the (**Figures 10 & 11**), the expression levels of *Caspase3*, *Caspase9* and *Bax* in both cell lines, exposed to all study groups were higher than the control group ($P<0.001^{***}$) and the expression levels of *Bcl2*, *MMP2* and *MMP9* in both cell lines, exposed to all study groups were lower than the control group ($P<0.001^{***}$). As can be seen in (**Figure 10& 11**), the administration of Melittin loaded niosome shows the synergic and higher up-regulating effects in (*Bax*, *Caspase3* and *Caspase9* genes) and down-regulating (*Bcl2*, *MMP2*, and *MMP9* genes) in both cell lines.

The apoptotic rates of human thyroid cancer cell line (TT) cells were increased following Melittin treatment, Melittin causes increased *Caspase3*, *Caspase9*, *Bax*, and inhibited B-cell lymphoma 2 genes and protein expression [59].

Melittin decreased the invasion rate of MCF-7 human breast cancer cell line by down-regulating CD147 and MMP-9 by inhibiting cyclophilin A expression so the results provide an evidence for Melittin in anticancer therapy and mechanisms [60].

Honey bee venom contain Melittin and chrysin are effective for destroying chemoresistant ovarian cancer cells through up-regulation of *Caspase3* and *Caspase9* and down-regulation of *Bcl2* genes expression[61].

3.8 Weight and volume changes

On the day before commencing all animals had same weight (about 19 ± 0.20 gr) and tumor volume (3 mm^3) the treatment and last day of the experiment, mice weights and tumor volume showed differences between groups (**Table 4**). Mice weight showed changes in the Melittin and Melittin loaded niosome groups compare to cancer and healthy controls. According to results, Melittin 6mg/kg and Melittin loaded niosome 3mg/kg control and treatment groups shows increase in mice weight and in compared to all groups the Melittin loaded niosome showed the largest increase. Tumor Volume showed changes in all treatment groups on the last day. The Groups receiving Melittin loaded niosome 3mg/kg, Melittin loaded niosom 1.5mg/kg, Melittin 6mg/kg and Melittin 3mg/kg showed a decrease in tumor volume compared to the control, respectively. Totally, Melittin loaded niosome 3mg/kg showed the greatest effect in inhibiting tumor growth and weight loss in mice.

3.9 Histopathology

Histopathology evaluation is

Score of malignancy in the histopathological view according to the results of histopathological studies of nuclear pleomorphism, the following scoring was evaluated: 0 = No pleomorphism / 1 = Small, regular nuclei and a shape / 2 = Moderate degree of difference in size and shape of the nucleus and hyperchromatic of the nucleus with the presence of nuclei / 3 = Severe degree of difference in nucleus size with hyperchromatic nuclei and often with one or more nuclei identified. Subsequently, the mitosis index was evaluated in 10 fields with a magnification of 40 (HPF) and the invasion index of the tumor cells was obtained and the following divisions were obtained:

0 = no mitosis / 1 = 9-1 mitosis / 2 = 10-19 mitosis / 3 = more than 20 mitosis

0 = absence of tumor cells in dermis and hypodermis / 1 = penetration into dermis / 2 = infiltration into hypodermis / 3 = penetration into subcutaneous muscle tissue (**Table 5**).

In the cancer control group extremely invasive tumor cells in the hypodermis and underlying muscle layer, severe necrosis at the center of the tumor, severe pleomorphism and severe hyperchromasia with high mitosis. The results indicate Necrotic cells, represent mitotic cells and severe cell polymorphisms (a). In healthy control, there are not any cancer cells and tissue in derma (b) and the group, with Melittin 3mg/kg treatment, there are few tumor cells that they are associated with inflammatory cells (c). There is tumor mass beneath the skin, indicates necrotic tissue and shows the prominent nuclear polymorphism (d) that was exactly in line with the results of the group with 3 mg/kg of Melittin group. Examination of mammary gland tissue sections that treated with 1.5mg/ kg of Melittin loaded niosome show a few numbers of tumor cells are found with very low cellular polymorphisms, and fewer inflammatory cells (e). Evaluation of breast tissue sections that treatment with 3 mg/kg Melittin loaded niosome show a few numbers of tumor cells are found in the skin hypodermis with very low cellular polymorphisms, and fewer inflammatory cells (f) (**Figure 12**).

Evaluated the efficacy of Melittin-loaded nanoparticles in immunocompetent mice. Syngeneic B16F10 mouse melanoma tumors in C57BL/6 mice actively secrete angiogenic growth factors and have already developed a vascular supply [62]. we have demonstrated that synthetic nanoscale vehicles such as nanoparticles can deliver a model cytolytic peptide (Melittin) by flexible passive and active molecular targeting to kill established solid tumors and precancerous lesions [9].

3.10 Evaluation of renal and liver enzyme activity

Determination of renal serum biomarker including BUN and Creatinine the groups treated with Melittin and Melittin loaded niosome compared to the healthy control group did not show significant changes. While the groups treated with Melittin loaded niosome 3mg/kg ratio compared to healthy control group showed higher effect than another groups. In the study of serum liver enzymes (AST, ALT, ALP, Albumin and Total Protein), there are not any changes in all groups compare to control.

Chronic 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) feeding in mice increased the serum levels of AST, ALT, AP and bilirubin. Of particular interest, the study showed that treatment with Melittin appeared to decrease AP and bilirubin concentrations in the serum of DDC-fed mice. Elevations of serum AST, ALT, AP and bilirubin, from liver metabolic disorder, play important roles in the initiation of liver fibrosis, and liver metabolic disorder was affected by pro-inflammatory cytokines that Melittin inhibited these disorders [63]. Melittin is beneficial for the prevention of acute hepatic failure in antitubercular drug-induced hepatotoxicity through regulate of AST,ALT, ALPBUN and Total bilirubin [64].

3.13 Immunohistochemical assay *P53*

According to the results of immunohistochemical assay studies, *P53* was not expressed as a prognostic factor in mammary gland carcinoma in all groups and was negative (**Figure 13**). Accumulated *P53* in node-negative breast cancer is associated with an aggressive course and a poor prognosis. Our result shows that *P53* accumulation cannot predict breast cancer in this study. A larger study is needed to further investigate the role of another factor as an independent prognostic factor in breast cancer. Taken together, these findings present new possibilities for Melittin treatment in tumor therapy. Although further studies are required to understand the detailed mechanisms interactions, the data presented here indicate that Melittin can be used as an anticancer therapy and represents a promising new approach.

Conclusion

The presented study was aiming for adequate, more effective, and less harmful breast cancer treatment. Our study successfully declares that Melittin-loaded nano-Niosome properly functions under the ideal-estimated status. This project has demonstrated that Niosomes are suitable vesicle carriers for combining drugs, particularly Melittin. Melittin loaded Niosome reported had strongly higher anti cancer effects in-vivo and in-vitro than free Melittin. Hopefully, this formulation can be industrialized in the next few years and reduces the breast cancer incidence rate.

Materials And Methods

2.1 Chemicals

Melittin was bought from Sigma Aldrich (Germany). Cholesterol, Span60, Tween60, and Dialysis membrane (MWCO 12kDa) were bought from Sigma Aldrich (USA). Chloroform, Amicon (Ultra15-Membrane, MWCO 30kDa), and Dimethyl sulfoxide (DMSO) were purchased from Merck (Germany). Medium RPMI-1640, DMEM (Dulbecco's Adjusted Eagle Medium), Trypsin-EDTA, Trypan blue, Fetal Bovine Serum (FBS), Phosphate-buffered saline (PBS), MTT (dimethylthiazol-2-yl)-2,5, and Penicillin / Streptomycin (PS)100 X were attained from Gibco, (USA). Pasteur Institute, Iran, provided the 4T1 murine mammary carcinoma and SKBR3 human mammary carcinoma. Annexin V-FITC Flow Cytometry kit was obtained from Affymetrix biosciences (USA). Transgene Biotech, China (Cat No. ER101-01 and AE301-02) has traded RNA Extraction and cDNA Synthesis kits. Pars Azmoon kit (Iran) to estimation of liver and renal biomarkers activity

2.2 Melittin loaded Niosomes Preparation

"Thin-layer hydration method" prepared the drug-loaded Niosomes containing cholesterol, span60 and tween60, reported in our previous work with minor modifications [27, 30]. Briefly, surfactants and cholesterol, were dissolved in 10 mL chloroform as organic solvent. A rotary evaporator (120 rpm, 60 °C, one hour) was used to evaporate the chloroform (Heidolph Instruments, Germany). Then, the dried thin films were hydrated utilizing Melittin solution (in PBS, 10 mL, pH 7.4) at 60 °C for one hour (120 rpm). Finally, the sample was sonicated for 5 min (Hielscher up50H ultrasonic processor, Germany, Amplitude: 25%, 200 w) to obtain the niosomal formulations with uniform size distribution (**Table 1**). The samples were kept in a refrigerator (4 °C) for further experiments.

2.3 Morphology, Size and Polydispersity of Index Measurements

Malvern Zeta Sizer (Malvern Instrument Ltd. Malvern, UK) was applied to distribute the size and polydispersity index based on dynamic light scattering (DLS). To investigate the optimum formulation morphology, transmission electron microscopy (TEM) at 80 KV (Netherland, Philips CM30), scanning electron microscopy (SEM) (SSX-500, Shimadzu, Japan) were utilized.

2.4 Determination of Encapsulation Efficiency (EE)

The Niosomes were ultra-filtered for 20 min at 4000×g, utilizing an Amicon. During filtration, free drugs moved through the filter membrane, and the Niosomal Melittin remained in the top chamber. Drug concentration at a wavelength of maximum absorbance peak of the drug molecule was analyzed by UV/Visible spectroscopy (JASCO, V-530, Japan), and drug concentration was evaluated according to its standard curve. Finally, the encapsulation efficiency estimated using the following equation:

$$\text{Encapsulation Efficiency (\%)} = [(A-B)/A] * 100$$

In this equation, "A" is the amount of initial drug trapped into the Niosomal formulations, and "B" stands for non-Niosomal loaded drugs released from the membrane.

2.5 In-Vitro Drugs Release Kinetic Study

The In-Vitro Melittin release from Niosomes was analyzed through the following method. Briefly, 2 ml of each sample was added to a dialysis bag. The dialysis bag with each sample was put in PBS solution (pH = 5.4, 6.5, 7.4) and stirred at 37 °C (50rpm). Then Aliquots were taken at specified intervals and the medium was refreshed. Several kinetic models were investigated and analyzed the release profile.

2.6 Niosome Stability studies

The stability was assessed by keeping the optimum formulation containing the Niosomal Melittin formulation in two different storage conditions (25 ± 1°C (room temperature) and 4 ± 1°C (refrigeration temperature) / 60 % RH ± 5 % RH) for two months. The physical properties accounted for vesicle size (nm), polydispersity index (PDI), and entrapment efficiency (%) was evaluated at certain time intervals (0, 14, 30, and 60 days).

2.7 Cells culture

The mouse breast epithelial 4T1 and SKBR3 cell line purchased from Pasteur Cell Bank, Iran. RNA. The cell line was cultured at 37 °C and in a 5% CO₂ in air atmosphere. All cells tested negative for mycoplasma contaminations and were markedly cultured in fresh medium (RPMI1640) supplemented with 10% fetal bovine serum (FBS, DENAzist Asia's C₀) and 1% antibiotics (penicillin/streptomycin). The cells (1×10^6 cells/ml) were plated in T-25 flasks containing 5 mls of RPMI1640 and grown in a humidified incubator under an atmosphere of 95% air and 5% CO₂ at 37 °C to sub confluence (90 - 95%). The culture medium was replaced every 48 hours. Once the cells reached 90 - 95% confluency, the medium was aspirated, and the cells monolayer was washed three times with sterile phosphate buffered saline. The cell monolayer was treated with 1 ml of 0.25% (w/v) trypsin-EDTA and incubated briefly at 37 °C and visualized microscopically to ensure complete cell detachment. Cells were re-suspended in complete growth medium. Cells were also stained with trypan blue (100 µl of cell suspension and 100 µl of 0.4% trypan blue), incubated for 2 minutes at room temperature, and counted using a hemocytometer.

2.8 Cell proliferation assay

4T1 and SKBR3 cell lines were separately seeded in a 96-well plate at a density of 5×10^5 /well including RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and 2% L-glutamine. Cells incubated under an atmosphere of 95% air at 37°C and 5% CO₂ for 24 h reaching 70-80% confluence. 8, 16, 32, 64, 128 and 256 µg/ml concentrations of Melittin and Melittin loaded niosomes were added and cells were incubated for 48 and 72 h. Incubation of the cells with 0.5 mg/ml of MTT was performed for 4 h followed by replacing the medium with 100 µl of DMSO and vortexing for 20 min. A microplate reader was used to measure the absorbance (570 nm). Median inhibitory concentration (IC₅₀) was calculated for samples.

2.9 Soft agar colony assay

4T1 and SKBR3 cells treated with Melittin and Melittin loaded niosome were suspended in 0.35% agarose and RPMI 1640 supplemented with 20% FBS and seeded over a basal layer of 0.5% agarose. The experiments were established in 100 mm petri dishes at a cell density of 6×10^3 cells/well. Colonies were manually counted in nine random fields after 21 days of culture at 37 °C. Phase contrast micrographs of the colonies were captured with a olympus (Tokyo, Japan).

2.10 Wound healing assay

To study cell migration, 4T1 and SKBR3 cancer cells were seeded at 5-105 cells/well in 24-well plates and incubated until they reached 70% confluence. Monolayers were scratched with a 200 µl pipette tip to create a wound, and cells were then washed twice with serum-free culture media to remove floating cells. Media were replaced with fresh serum-free medium. Cells were subjected to the indicated treatment with 8, 16, 32, 64, 128 and 256 µg/ml concentrations of Melittin and Melittin loaded niosomes in medium for 72 hours. Then Cells were washed with PBS, fixed and microscopic photos were made.

2.11 Flow cytometric analysis

To evaluate the apoptosis/necrosis ratio, the 4T1 and SKBR3 cells were treated with Melittin and Melittin loaded niosomes IC₅₀ concentration for 72 h, and then the cells were studied using Annexin V/propidium iodide (PI) assay, according to the manufacturer's instructions. Briefly, cells were two times washed using cold PBS followed by resuspending in 1X binding buffer (5×10^5 cells/ well). A culture tube (5 ml) was then filled with 100 µl of the solution. The tubes were then filled with FITC Annexin V and PI (each 5 µl). A gentle vortex was considered followed by incubation (25°C/15 min) in the darkroom. The tubes were provided by 400 µl of 1X binding buffer. Flow cytometry was applied for analysis for 1h. The cells without any treatment were used as control. Finally, the levels of apoptotic/necrotic cells were evaluated using flow cytometry.

2.12 Gene expression analysis by Real-Time PCR

2.12.1 RNA extraction

Up to 1×10^7 cells were cultured in T25-flasks and treated with different concentrations M and NM IC₅₀ concentration for 72 h. Add 1 ml ice-cold RNX TM – PLUS solution to 2 ml then tube containing homogenized sample. Vortex 5-10 second and incubate at

room temperature for 5 min. Add 200 µl of chloroform. Mix well for 15 second by shaking. Incubate on ice or 40C for 5 min. Centrifuge at 12000 rpm at 40C for 15 min. Transfer the Aqueous phase to a new RNase-free 1.5 ml tube, (do not disturb the mid-phase) and add an equal volume of isopropanol. Gently mix and incubate ice for 15 min. Centrifuge the mixture at 12000 rpm at 40 °C for 15 min. Discard the supernatant and add 1 ml of 75% Ethanol, shortly vortex to dislodge the pellet and then centrifuge at 4 °C for 8 min at 7500 rpm. Discard the supernatant and let the pellet to dry at room temperature for a few minutes (do not let dry completely, it will decrease the solubility of the pellet. Dissolve pellet in 50 µl of DEPC water. To help to dissolve, place the tube in a 55-60 OC water bath for 10 min. Total RNA was extracted using Tripura reagent according to the manufacturer's instruction.

2.12.2 cDNA synthesis

Mix the template RNA (total RNA or Poly (A) mRNA) 1ng~5µg, Buffer-Mix (2X) 10 µl, Enzyme-Mix 2 µl and DEPC-treated water up to 20 µl components in RNase-free tube. Mix the above mixture by quick vortex then incubate 10 min at 250C. Incubate 60 min at 470C. Stop the reaction by heating at 850C for 5 minutes. Chill on the ice or at 40C. To perform PCR, you can add the finished RT reaction up to 1.5 of the final PCR volumes.

2.12.3 Primer design and RT-PCR

The particular primers for *Drp1*, *Mfn1*, *Bax*, *Bcl2*, *Caspase3*, *Caspase9*, *MMP2*, *MMP9* and *β-actin* (as internal control) were designed through the National Center for Biotechnology Information (NCBI) website (**Table 2**).

2.12.4 Quantitative Real Time PCR

Experimental reaction by was performed by adding the components in this order, RealQ Plus and 2X Master MIX (12.5 µl Vol./reaction, 1X), Primer A 10µM (0.5 µl (0.25 – 2.5 µl) Vol./reaction, 0.1 µM (0.05 – 0.5 µM)), Primer B 10µM (0.5 µl (0.25 – 2.5 µl) Vol./reaction, 0.1 µM (0.05 – 0.5 µM))), PCR-grade H2O (10.5 µl Vol./reaction), template DNA (1µl) and total volume is 25 µl. Then gently mixed without creating bubbles. The reaction microtubes were placed in the instrument and the appropriate program according to the manufacturer's instructions was run, (1 Cycles, 15 minutes Duration of a cycle, 95 OC Temperature) and (40 Cycles, 30 seconds duration of cycle in 95 OC Temperature and 30 seconds duration of cycle in 61.5 OC Temperature).

2.13 In-vivo study

2.13.1 Experimental Animals and Ethical aspects

All animal care and experimental procedures were carried out strictly following the criteria of the Institutional Animal Care and Use Committee of Kharazmi University- (Laboratories' Animal Center & Cellular and Molecular Research Laboratory, Kharazmi University, Tehran, Iran/ (ethical code: 16/11/2018-616/9745). All efforts were made to minimize the animals' suffering and the numbers used. In this study 35 females, BALB/c inbred mice (weighing 18±2 g, 6 to 8 weeks) were individually housed in polycarbonate animal cages and maintained under constant temperature (22 ± 2°C) and humidity (55%). The mice had free access to food and water and were kept in 12 h light-dark cycles. After 1 week of acclimatization, the BALB/c mice were randomly divided into seven groups (*n* = 5 per group).

2.13.2 Induction of Breast cancer

The experimental breast tumor was induced by subcutaneous injection of 4T1 tumor cells (105 /mL in suspension with phosphate-buffered saline, PBS 1X). The injection site was on the right side of the chest pad. 12th day after tumor induction, a solid tumor appeared subcutaneously. After 2 weeks, tumors were palpable. Tumor volume and mice weight was measured by using a digital scale and a digital caliper and calculated according to Formula 1 and formula 2, mentioned below;

Formula 1: Volume = (width 2) x length/2

Formula 2: Total Volume (mm3) = volume at last day (mm)/ volume at first day (mm) × 100

2.13.3 Study design

Two doses of Melittin loaded niosome and LD50 dose of Melittin were considered in treatment groups. Daily intraperitoneally (i.p.) injection of Melittin for 20 days was performed. Healthy animals were divided into seven experimental groups of 5 mice each:

Gr.1 - mice treated intraperitoneally (i.p.) with Melittin at a dose of 3 mg/kg.

Gr.2 – mice treated intraperitoneally (i.p.) with Melittin at a dose of 6 mg/kg

Gr 3 - mice treated intraperitoneally (i.p.) with Melittin loaded niosome at a dose of 1.5mg/ kg.

Gr.4 - mice treated intraperitoneally (i.p.) with Melittin loaded niosome at a dose of 3 mg/ kg.

Gr.5 - mice treated intraperitoneally (i.p.) with PBS.

Gr.6 - Cancer control

Gr.7 - Health control

2.13.4 Estimation of enzyme activity

The end of the study period, animal blood samples were taken then liver and renal biomarkers were measured. The level of activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Albumin, Creatinine, blood urea nitrogen (BUN), Total Protein in the collected samples was determined spectrometrically using “Pars Azmoon kit.”

2.13.5 Histopathology

Samples LD50 were considered in treatment groups, daily intraperitoneally (i.p.) injection of samples for 20 days were performed. Three mice of each group were sacrificed at the end of the treatment period. The tumor mass was removed and fixed in 10 % buffered formalin. Tumor tissues were processed in a tissue processor, and paraffin-embedded tissue sections were stained by hematoxylin and Eosin (H&E) method. The tumor was histologically classified according to the Nottingham histologic score system (Menten grade) [31]. By this scoring system, the amount of nuclear features, gland formation, and mitotic activity was assessed. All methods used in the study comply with the institutional ethical guidelines for care and the use of animals in research.

2.13.6 Immunohistochemical analysis of the tumors

Tumor tissues were fixed in 10% formalin buffer solution, washed three times in PBS, and left in 70% ethanol. Tumors were kept in paraffin, and 5-µm sections were prepared. For Hematoxylin and Eosin stainin, slides were dewaxed, hydrated using a decreasing solution bank of ethanol, stained with Gill'sg hematoxylin, dehydrated using 70% ethanol, stained with eosin, further dehydrated using 100% ethanol, cleared using toluene, and mounted in coverslips using Acrymount IHC mounting media (StatLab). Tumor cells apoptosis were determined in tissue sections by TUNEL assay (In Situ Cell Death Detection Kit, Roche).

2.14 Statistical analysis

Data were reported as mean $\pm$ SD and the graphs were plotted using GraphPad Prism version 8. Data were statistically analyzed using analysis of variances (ANOVA) followed by post-Tukey test and a p-value less than 0.05 was considered as a significant difference.

Declarations

Ethics approval and consent to participate

There are no “human subjects” in this study

Consent for publication

Not applicable

Availability of data and materials

All data were analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

F.D.M., I.A. and P.M. developed the idea and designed the experiments. F.D.M., I.A., E.M., M.F., L.K., and M.J. conducted the experiments. F.D.M. and A.R. analyzed the data. F.D.M. and A.R. wrote the manuscript. All authors confirmed the final manuscript before the submission.

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Tables

Table 2. Effect of the surfactant: cholesterol and span 60: tween 60 with various molar ratios on entrapment efficiency (EE %), size and PDI in Melittin loaded niosome.

Formulations	Surfactant: Cholesterol (molar ratio)	Span60:Tween60 (molar ratio)	Drug concentration (mg/ml)	Sonication time (min)	Vesicle Size (nm, average $\pm$ SD)	Polydispersity index (average $\pm$ SD)	EE (%) (average $\pm$ SD)
T ₁	1:1	100:0	1	5	197.4 $\pm$ 8.3	0.175 $\pm$ 0.024	60.25 $\pm$ 1.75
T ₂	1:1	50:50	1	5	121.4 $\pm$ 8.7	0.211 $\pm$ 0.012	79.32 $\pm$ 1.34
T ₃	1:1	0:100	1	5	158.4 $\pm$ 6.5	0.192 $\pm$ 0.021	67.21 $\pm$ 0.92
T ₄	2:1	100:0	1	5	235.7 $\pm$ 10.1	0.254 $\pm$ 0.016	51.33 $\pm$ 2.78
T ₅	2:1	50:50	1	5	165.3 $\pm$ 9.2	0.184 $\pm$ 0.008	75.45 $\pm$ 2.34
T ₆	2:1	0:100	1	5	208.9 $\pm$ 5.6	0.224 $\pm$ 0.017	64.75 $\pm$ 1.52

Table 3. The kinetic release models and the parameters obtained for optimum niosomal formulation.

Release model	Melittin solution	Melittin loaded niosome (pH 7.4)	Melittin loaded niosome (pH 6.5)	Melittin loaded niosome (pH 5.4)
Zero-Order $Q_t = Q_0 + K_0 t$	R ² =0.5679	R ² =0.7368	R ² =0.7698	R ² =0.7784
First-Order $\text{Log } Q_t = \text{Log } Q_0 + K_1 t$	R ² =0.9643	R ² =0.8319	R ² =0.8833	R ² =0.9380
Higuchi $Q_t = Q_0 + K H t_{0.5}$	R ² =0.7415	R ² =0.8845	R ² =0.9073	R ² =0.9140
Korsmeyer-Peppas $\frac{M_t}{M} = k t^n$	R ² =0.8337 n=0.4435	R ² =0.9003 n=0.4454	R ² =0.9297 n=0.4141	R ² =0.9431 n=0.4021

Table 4. Mice weight and tumor volume changes after 20 days of treatment

Groups	Last day weight	Last day tumor volume
Cancer Control	15.50	0
Healthy Control	21.93	6.01
Melittin 3mg/kg	20.12	5.12
Melittin 6mg/kg	20.69	4.78
Melittin loaded niosome 1.5mg/kg	21.13	3.22
Melittin loaded niosome 3mg/kg	21.52	3

Table 5. Clinico-Pathological Result

Group	Pleomorphism	Mitosis index	Invasion
Cancer Control	3	3	3
Healthy Control	0	0	0
Melittin 3mg/kg	3	3	2
Melittin 6mg/kg	2	2	1
Melittin loaded niosome 1.5mg/kg	2	2	2
Melittin loaded niosome 3mg/kg	2	1	1

Table 6. Evaluation of serum parameters in study groups.

Group	BUN	Creatinine	AST	ALT	ALP	Albumin	Tolat Protein
Healthy Control	21.35	0.43	215.4	110	295	2.95	4.95
Melittin 3mg/kg	19.22	0.5	218	115	310	2.87	4.95
Melittin 6mg/kg	21	0.44	216.11	113.13	301	2.9	4.92
Melittin loaded niosome 1.5mg/kg	21.13	0.51	214.14	109.11	294	2.9	4.98
Melittin loaded niosome 3mg/kg	21.02	0.43	213	111.2	290	2.89	4.9

Table 2. The used primer sequences in real time polymerase chain reaction

Genes	Forward primer (5'-3')	Reverse primer (3'-5')
<i>Bax</i>	CGGCAACTTCAACTGGGG	TCCAGCCCAACAGCCG
<i>Bcl2</i>	GGTGCCGGTTCAGGTACTCA	TTGTGGCCTTCTTTGAGTTCG
<i>Caspase3</i>	CATACTCCACAGCACCTGGTTA	ACTCAAATTCTGTTGCCACCTT
<i>Caspase9</i>	CATATGATCGAGGACATCCAG	TTAGTTCGCAGAAACGAAGC
<i>MMP2</i>	TTG ACG GTA AGG ACGGAC TC	CAT ACT TCA CAC GGA CCA CTTG
<i>MMP9</i>	GCACGACGTCTTCCAGTACC	CAGGATGTCATAGGTCACGTAGC
<i>β-actin</i>	TCCTCCTGAGCGCAAGTAC	CCTGCTTGCTGATCCACATCT

Figures

Figure 1

Morphological determination of optimized formulation. A: Analysis of particle size distribution of Melittin loaded niosome by Dynamic Light Scattering (DLS); B: Scanning electron microscopy (SEM); C: Transmission electron microscopy (TEM).

Figure 2

A comparison between In-Vitro drug release profile of Melittin and Melittin loaded niosome from the dialysis bag in different pH (7.4, 6.5 and 5.4) at 37°C.

Figure 3

Comparing Stability of optimum formulation at 4°C and 25°C. Mean particle size (a), PDI (b) and EE % (c) were studied as stability parameters. Results are represented by mean $\pm$ SD (n = 3). *p < 0.05, ***p < 0.001.

Figure 4

Melittin hemolytic activity compared with the positive control (Triton X-100). All experiments were performed in triplicate. Data is shown as mean $\pm$ SEM. The mean values with different superscript letters are significantly different ($P \leq 0.05$).

Figure 5

IC₅₀ value in study groups after 48 and 72 h treatment with Melittin and Melittin loaded niosome ($P < 0.001$ ***) on 4T1 cell line (a). IC₅₀ value in study groups after 48 and 72 h treatment with Melittin and Melittin loaded niosome ($P < 0.001$ ***) on SKBR3 cell line (b). IC₅₀ value in study groups after 48 and 72 h treatment with with Melittin and Melittin loaded niosome on 4T1 cells with each other ($P < 0.001$ ***) (c). IC₅₀ value in study groups after 48 and 72 h treatment with Melittin and Melittin loaded niosome on SKBR3 cells with each other ($P < 0.001$ ***) (d). Data is shown as mean $\pm$ SEM. The mean values with different superscript letters are significantly different ($P \leq 0.05$).

Figure 6

The Figure shows the cell viability percent of different Niosome dilution on MCF10A cells (a) and cell viability percent of Melittin loaded niosome concentrations on healthy MCF10A cell line (f). Data is shown as mean $\pm$ SEM. The mean values with different superscript letters are significantly different ($P \leq 0.05$).

Figure 7

The inhibitory effects of Melittin and Melittin loaded niosome on the migration and invasion of the SKBR3 (a) and 4T1 breast cancer cells (b) after 72h of treatment; Data is shown as mean $\pm$ SEM. The mean values with different superscript letters are significantly different ($P \leq 0.05$).

Figure 8

The number of colonies was concentration-dependently decreased by Melittin and Melittin loaded niosome compared to control (Figure 8). Number of colonies in SKBR3 cell line was decreased by treatment with Melittin loaded niosome compared to Melittin ($P<0.001^{***}$) (Figure 8a) and in 4T1 cell line the results was same ($P<0.001^{***}$) (Figure 8b).

Figure 9

The flow cytometric analysis diagram of Melittin and Melittin loaded niosome provided in SKBR3 (Figure 9a) and 4T1 breast cancer cell lines (Figure 9b).

Figure 10

The expression levels of Caspase3 (a), Caspase9 (b), Bax (c), Bcl2 (d), MMP2 (e) and MMP9 (f) genes treated by Melittin loaded niosome and Melittin in SKBR3 cell lines. Data is shown as mean $\pm$ SEM. The mean values with different superscript letters are significantly different ($P \leq 0.05$).

Figure 11

The expression levels of Caspase3 (a), Caspase9 (b), Bax (c), Bcl2 (d), MMP2 (e) and MMP9 (f) genes treated by Melittin loaded niosome and Melittin in 4T1 cell lines. Data is shown as mean $\pm$ SEM. The mean values with different superscript letters are significantly different ($P \leq 0.05$).

Figure 12

Microscopic views of malignant mammary tumors. H&E staining of cancer control (original magnification, x400) (a); H&E staining of healthy control (original magnification, x400) (b); H&E staining of Melittin 3mg/kg (original magnification, x400) (c); H&E staining of Melittin 6mg/kg (original magnification, x400) (d); H&E staining of Melittin loaded niosome 1.5mg/kg (original magnification x400) (e); H&E staining of Melittin loaded niosome 3mg/kg (original magnification, x400) (f). (N: Necrosis / Arrowhead: Polymorphism / Arrow: Cancer tissue and cell mitosis).

Figure 13

Immunohistochemistry staining of P53 in mammary gland carcinoma in all experimental groups. Cancer control (original magnification, x40) (a); Healthy control (original magnification, x40) (b); Melittin 3mg/kg (original magnification, x40) (c); Melittin 6mg/kg (original magnification, x40) (d); Melittin loaded niosome 1.5mg/kg (original magnification x40) (e); Melittin loaded niosome 3mg/kg (original magnification, x40) (f).

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