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Administration of Apis cerana cerana melittin gene-encapsulated pectin for breast cancer therapy: an investigation of a new anti-cancer agent

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Abstract

Purpose: One intriguing therapy option for cancer is the peptide *Melittin*. This study assessed the in vitro anticancer effects of *Melittin*, *Melittin*-loaded pectin, and free pectin on mammary cell lines.

Methods: The "thin-layer hydration approach" produced pectins; various *Melittin* pectinal compositions were created and analyzed for morphology, dimensions, polydispersity index, entrapment efficiency, release kinetics, and stability. The breast cancer cells were evaluated with the hemolytic activity assay, flow cytometry, the MTT assay, and wound healing assessment. Real-time PCR was used to assess the transcription of the genes ($P < 0.05$).

Results: This study indicated that *Melittin*-loaded pectin is a suitable alternative for breast cancer therapy owing to enhanced targeting, encapsulation efficacy (81.43 ± 1.43), PDI (0.207 ± 0.43), release percentage, and a notable anticancer impact on cell lines. *Melittin*-loaded pectin significantly affects gene transcription in the cells under study compared to other samples; it down-regulates the transcription of the *BCL2*, *MMP2,* and *MMP9* genes ($P < 0.05$) while up-regulating the expression of the *Bax, P57*, *caspase3*, and *caspase9* genes.

Conclusions: This study has shown that *Melittin* plasmid-loaded pectin exhibits superior anticancer activities compared to free *Melittin*. This research demonstrates that, unlike its free form, pectins are suitable vesicle transporters for *Melittin*.

Keywords: Nanotechnology, Pectin, Cancer, *Melittin*

Introduction

Breast cancer is the second leading cause of cancer death for female patients, accounting for 30% of all new instances of cancer women (DeSantis et al. 2019). The American Cancer Society forecasts that over 42,000 individuals will succumb to breast cancer this year, notwithstanding advancements in mortality patterns, and the 5-year survival rate for breast cancer with metastatic spread is 27% (Rahib et al. 2014). This indicates

that more efforts are required in the treatment of advanced breast cancer. The progression and recurrence of the illness substantially affect the prognosis of breast tumors in patients (DeSantis et al. 2019; Rahib et al. 2014; Waks et al. 2019). Because breast cancer is difficult to cure and is easy to infiltrate and spread, it is imperative to find safe and effective therapeutic drugs to battle it (Costa et al. 2020; Piri-Gharaghie et al. 2022). The development of innovative antimitotic agents represents an effective chemotherapeutic strategy for the treatment of breast cancer. Because tubulin plays a crucial role in mitotic spindles, antimitotic drugs that change tubulin dynamics may prevent cell division and cancer progression (Bates et al. 2017).

The therapeutic methods for breast cancer treatment depend on the tumor's distinct biological properties (Bates et al. 2017). Hormone therapy may be recommended for poor-quality, node-negative, and estrogen receptor-positive tumors (Burstein et al. 2014). Chemotherapy is frequently utilized prior to targeted treatments when the tumor has an advanced stage and is node-positive, contingent upon the hormonal/ErBb2 status of the tumor (Talima et al. 2019). The existing protocols for breast cancer treatment include anthracyclines, including taxanes, doxorubicin, and epirubicin (Khasraw et al. 2012). Nonetheless, substantial hurdles persist in the fight against breast cancer, and the development of novel pharmaceuticals that may be integrated with current therapies to amplify their efficacy and mitigate their side effects would be very advantageous. Due to their straightforward production, high potency, and cell selectivity, anti-cancer peptides (ACPs) have emerged as a promising category of cancer therapies (Malik et al. 2022). ACPs demonstrate their anti-tumor efficacy via several mechanisms, including the selective targeting of tumor-suppressive signaling pathways and the induction of cell death by membrane lysis (Mukherjee et al. 2023). When applied off-target, ACPs exhibit more excellent selectivity and less toxicity to normal cells than conventional chemotherapeutics (Hilchie et al. 2019). Crucially, due to the impact of ACPs on essential cellular activities that are unavoidable for cells, the likelihood of cancer cells developing drug resistance against ACPs is negligible (Hilchie et al. 2019). The recurrent susceptibility of malignant cells to ACPs is a notable advantage of ACP, mainly when drug resistance is often responsible for the failure of chemotherapy (Diwan et al. 2022). The development of ACP delivery systems has garnered increased interest owing to the attractive attributes of ACP (Hilchie et al. 2019; Diwan et al. 2022).

Extensive research has examined several approaches to enhance the accessibility of ACPs to malignant cells (Hilchie et al. 2019; Diwan et al. 2022; Johnson et al. 2016). Researchers found that the EGFR(2R)-lytic-peptide exhibited enhanced binding capacity to EGFR when the following amino acid, histidine (H), was substituted with arginine (R). The cationic amphipathic polypeptide KLA (KLAKLAKKLAKLAK) induces programmed cell death by disrupting the mitochondrial membrane; nevertheless, its inadequate penetration of cancer cells significantly limits its effectiveness and application (Hyun et al. 2014). The Apis cerana cerana melt gene for *Melittin* (GIGAVLKVLTTGLPALISWIKRKRQQ). This active peptide has been shown to have anti-tumor action against various cancers, including liver, bladder, and prostate (Ghaly et al. 2023). *Melittin* can react with and disrupt cellular membranes owing to its cationic properties and amphipathic nature (Guha et al. 2021). *Melittin* is a membranolytic compound that may modify many cellular signaling pathways. For instance, it may impede metastasis,

cause cell cycle arrest, and inhibit angiogenesis. Notwithstanding *Melittin*'s significant potential as a cancer treatment, several critical drawbacks hinder its use in clinical environments. These constraints include nonspecific hemolysis, rapid clearance and degradation, and inadequate tumor accumulation (Ghaly et al. 2023; Guha et al. 2021). To mitigate these issues, *Melittin* has been included in drug delivery systems, including polymer–peptide conjugates and nanoparticles (NP) (Lv et al. 2021; Guha et al. 2021). To The objective is to minimize nonspecific hemolysis to enhance in vivo safety and increase bioavailability in malignancies. Honey bee venom may enhance T lymphocyte activity and expression in mice with sarcomas. *Melittin* enhances T1 cell functions and addresses cancer, infections caused by viruses, and compromised immune function. *Melittin* has markedly reduced the capacity of cancer cells to metastasize and invade by inducing apoptosis and necrosis, blocking angiogenesis, and causing cell cycle arrest. *Melittin* suppresses the proliferation of 4T1 breast cancer cells by upregulating the genes for *Bax*, Mfn1, *Caspase3*, and *Caspase9* while downregulating the genes for *BCL2*, Drp1, *MMP2*, and *MMP9*, positioning it as a promising candidate for further research in breast cancer treatment. Integrating it with a more precise procedure is advantageous to enhance the efficacy of the therapy. Many natural compounds exhibiting anti-cancer properties and chemotherapeutic drug-conjugated nanoparticles have shown favorable outcomes and have been approved for treating many cancer types (Lv et al. 2021; Vimala et al. 2021).

Nanotechnology is an innovative science that considers the nanoscale's unique properties (Jones et al. 2013). Due to their significant surface area to mass ratio and often effective interactions with their surroundings, nanoparticles may function as restricted carriers for their constituent molecules instead of the same molecules in solution (Sapsford et al. 2013). Consequently, nanoparticles serve as effective delivery methods for therapeutic alternatives (Yetisgin et al. 2020). Nanoparticles have been engineered to optimize their dimensions and characteristics to enhance the biological distribution of cancer therapeutics in circulation (Mitchell et al. 2021). A further obstacle to the efficacy of targeted molecular chemotherapeutic drugs, which may restrict the use of nanoparticles, is drug resistance (Yao et al. 2020). The advancement of multifunctional and multiplex nanoparticles for oncological therapy is now a prominent research focus (Rahman et al. 2012). Formulations intended to transport medication to the specific site of action within the body are drug delivery systems. Pectin is a polysaccharide with the benefits of being non-toxic, biodegradable, and affordable (Khotimchenko et al. 2020). Such characteristics make it a potential choice for nanoparticle manufacturing. Polysaccharide-based nanoparticles, such as pectin, have garnered significant interest in oral medication delivery due to their exceptional solubility and mucoadhesive characteristics. Pectin has enough encapsulation ability, biocompatibility, biodegradability, low manufacturing cost, and appropriate stability, making it a viable alternative to phospholipid vesicles for encapsulating hydrophobic and hydrophilic pharmaceuticals (Lara-Espinoza et al. 2018).

Recent studies persist in investigating melittin's cytotoxic properties on several cancer cell lines, particularly those associated with breast cancer (Haque et al. 2023). Research has examined the processes of melittin, which include altering cellular membranes, obstructing signaling pathways, and triggering apoptosis. Nonetheless, a significant issue persists due to the non-specific toxicity of melittin, which may adversely affect healthy

cells as well (Lee and Bae 2016). Consequently, considerable efforts are directed toward creating tailored delivery methods to improve treatment effectiveness while reducing adverse effects. Recent studies have investigated melittin's synergistic effects in conjunction with other chemotherapeutic medicines or radiation therapy, with the objective of enhancing treatment efficacy and mitigating drug resistance (Zhang et al. 2024). Furthermore, research is exploring modified melittin analogs that have less toxicity while preserving anti-cancer efficacy (Akbarzadeh-Khiavi et al. 2022).

Pectin, a natural polysaccharide, has emerged as a potential substance for drug administration owing to its biocompatibility, biodegradability, and capacity to form hydrogels or nanoparticles. The mucoadhesive characteristics are especially beneficial for administering pharmaceuticals to mucosal surfaces (Li et al. 2021). Recent research has investigated pectin-based delivery methods for many anti-cancer agents, including melittin. These methods seek to safeguard melittin against degradation, regulate its release, and improve its delivery to tumor locations (Singh et al. 2023). Researchers have examined several pectin changes to enhance drug loading, stability, and targeting. Research has investigated enzyme-sensitive or pH-sensitive pectin-based carriers that release melittin selectively inside the tumor microenvironment (Sang et al. 2024).

The amalgamation of melittin and pectin has significant potential for breast cancer therapy. Pectin-based delivery methods may mitigate the toxicity constraints of melittin by encapsulating and targeting it precisely to breast cancer cells (Kaur et al. 2024). Recent investigations have concentrated on creating innovative pectin-based carriers, such as nanoparticles or hydrogels, infused with melittin (Ren et al. 2022). These studies have assessed the anti-cancer efficacy of these systems both in vitro and in vivo, evaluating their capacity to suppress breast cancer cell proliferation, decrease tumor size, and mitigate systemic toxicity. Research has investigated the use of targeting ligands conjugated to pectin to improve the delivery of melittin to breast cancer cells (Zaiki et al. 2023). Current research in this domain aims to enhance the design of pectin–melittin delivery systems to get superior treatment results for breast cancer patients.

The objectives of the current study were to investigate the administration of *Melittin* using pectinal formulations to enhance its stability and controlled release, thereby improving its anticancer efficacy, inducing apoptosis, inhibiting migration and invasion, and affecting breast cancer cells and BALB/c tumor mice.

Methods

Recombinant plasmids construction

Generay Biotechnology Co. (Shanghai, China) commercially manufactured the nucleic acid sequences and integrated them into the pcDNA3.1(+) (pDNA) Mammalian Expression Vector. The peptide and acid nucleic sequences were utilized, as specified in Table 1.

Table 1 Sequences of the *Melittin* peptides was used in this study

Symbole	Amino acid Sequence	Size (kD)	Nucleic acids Sequence
Apis cerana cerana melt gene for *Melittin*	MGIGAVLKVLTTGLPAL-ISWIKRKRQQ	2.97868	atgggaattggagctgtgctgaaggt-gctgacaacaggactgcccgccct-gatcagttggatcaagagaaagaga-cagcagtaa

The start codon was added at the beginning of the *Melittin* sequence. The recombinant *Melittin* acid nucleic was sandwiched by restriction enzymes BamHI (GGATCC) and EcoRV (GATATC). All plasmids were transformed into E. coli TOP10F utilizing the CaCl2 method and subsequently extracted using the Endo-free Plasmid Extraction kit (Favorgen, Taiwan). The cloning effectiveness was assessed through PCR, DNA sequencing, and endonuclease digestion assays. The subsequent antibiotic concentrations were employed to select the plasmids: 100 μg/mL ampicillin for *Escherichia coli*. Recombinant plasmids were preserved at – 20 °C.

Confirmation of the cloning of *Melittin* into the pDNA mammalian expression vector

Recombinant *Melittin* was monitored by a PCR process using specific primers. The following components are employed in a 25-mL PCR reaction: 12.5 μL of 2×PCR master mix (Yekta Tajhiz Azma, Iran), 0.5 μL (10 pmol) of each primer (Cinnagen, Iran), 3.5 μL (100 ng) of plasmid, and 8 μL H2O. The PCR temperature protocol consists of an initial annealing phase at 95 °C for 5 min, after which 30 cycles are performed at 94 °C for 50 s, 60 °C for 50 s, and 72 °C for 60 s. The last extension was executed after 10 min at 72 °C. A UVI Tech (England) gel imaging equipment was employed to analyze and document the bands after the electrophoresis of the PCR result on a 1% agarose gel containing a safe stain (Yekta Tajhiz Azma, Iran).

pDNA/*Melittin* loaded pectin preparation

We used the "Thin-Layer Hydration Technique" to create the pDNA/*Melittin*-loaded pectin (pDNA/*Melittin*/pectin). The pectin powder was dissolved in 10 mL of chloroform solution. A Heidolph Instruments rotary evaporator (120 rpm, 60 °C, 1 h) evaporated the chloroform. Subsequently, a recombinant pDNA/*Melittin* was dissolved in PBS (pH 7.4), and 10 mL of this solution was employed for hydrating the dried thin films at 60 °C for 1 h at 120 rpm. The sample was further 80% power sonicated for 15 min using an ultrasonic homogenizer-FAPAN400UPT1 model (iranpajoh, Iran) to produce pectinal components with a uniform size distribution. The solutions were preserved in a refrigerator at 4 °C for further analyses.

Analyses of the morphological characteristics, diameter, and polydispersity index

The size and polydispersity index were determined using dynamic light scattering (DLS) utilizing the Malvern Zeta Sizer (Malvern Instrument Ltd., Malvern, UK). The mean of triplicate examinations was employed to ascertain the diameter of each sample (measured in nanometers) and the surface charge. The physical properties of nanoparticles were analyzed using high-resolution transmission electron microscopy (Hitachi, HT7700 Exalens). SEM (SSX-500, Shimadzu, Japan) was employed to investigate the morphology of the optimal formulation.

Entrapment efficiency evaluation (EE)

The pectinal nanostructures were ultra-filtered using an Amicon for 20 min at 4000×g. The pDNA/*Melittin*/pectin was maintained in the top column, whereas free pectins floated through the filter membrane. The pDNA/*Melittin*/pectin concentration was determined by UV/visible spectroscopy (JASCO, V-530, Japan) at a wavelength, where

the drug molecule's most prominent absorbance peak was detected. The pDNA/*Melittin*/pectin concentration was then determined using its standard curve. The effectiveness of encapsulation was ultimately computed utilizing the equation:

$$\text{Entrapment efficiency (\%)} = [(A - B)/A] \times 100$$

In this equation, "A" denotes the original pDNA/*Melittin* concentration encapsulated inside the pectinal matrices, whereas "B" signifies the non-pectinal-loaded pDNA/*Melittin* concentration liberated from the membrane.

OliGreen (OG) fluorescent nucleic acid dye test

Complexes containing four nanograms of recombinant plasmid nanostructures were administered to each sample. Subsequently, 25 µL of 1X OliGreen (OG) fluorescent nucleic acid stain was introduced, and the mixture was allowed to incubate for 5 min at room temperature in the dark inside a 1.5 mL cuvette. After incubation, the total capacity of the cuvette was adjusted to 500 µL employing Millipore water. A luminescence spectrophotometer assessed the relative fluorescence employing 490 nm excitation and 520 nm emission wavelengths.

pDNA/*Melittin*/pectin release kinetics in vitro

The in vitro release of *Melittin* from pectins was investigated using the following methodology: 2 mL of each sample were promptly placed in a dialysis bag. A PBS solution (pH 5 and pH 7) was introduced into the bag, holding each sample, and agitated at 37 °C at 50 rpm. The medium was replenished following the collection of aliquots at specified intervals. A variety of kinetic models were analyzed, and the release pattern was investigated.

pDNA/*Melittin*/pectin stability research

The stability was evaluated by keeping the optimal combination, including the pectinal *Melittin*, for 2 months at two distinct temperatures [25 °C at ambient temperature and 4 °C at refrigeration temperature]. The physical characteristics, such as the vesicle size (nm), polydispersity index (PDI), and encapsulation effectiveness (%), were analyzed at certain time intervals (0, 15, 30, 45, and 60 days).

Cell culture

MCF-10A, SKBR3, and MCF-7 were kept in RPMI 1640 (Gibco, Thermo Fisher Scientific, United States) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, United States). Cells were seeded in a 96-well plate at an average of 15,000 to 20,000 cells per well. The cells were cultured in an environment with 5% CO2 at 37 C. 3×10^5 cells were transfected by transferring them to a 6-well plate until they achieved 75% confluence (24 h). According to the standard instructions provided by the manufacturer, LipofectamineTM2000 (Thermo Fisher Scientific, USA) was used to transfect the plasmids into cells. A cell culture medium containing 2 µg/mL Neomycin antibiotic was substituted for the media for transfected cell screening 24-h post-transfection.

Cell viability assay

The MTT technique was employed to assess the cytotoxicity of the different formulations on the viability of MCF-7 and SKBR3 cells. MCF-10A is utilized to simulate healthy human breast cells. To assess the survival of cells, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich) reagent was used. The different formulations with final concentrations of 40 µg/mL, 80 µg/mL, 120 µg/mL, and 240 µg/mL were added to the cell culture. After a 2 h of incubation at 37 °C, the culture medium was removed from the cells. MTT was then included in a DMSO (Sigma Aldrich) solution, and the resultant combination was assessed for absorbance at a wavelength of 570 nm.

The wound healing examination

MCF-7 and SKBR3 cancerous cells were seeded at 5×10^5 cells per well in 24-well plates and cultured until they reached 70% confluence to assess cellular motility. After creating a wound on monolayers using a 200 µl pipette tip, the cells were washed twice with serum-free culture media to eliminate suspended cells. A new serum-free medium was employed to replace the previous media. Cells were administered PBS, *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin at IC50 doses. After 18 h, PBS was employed to clean the cells, fix them, and capture microscopic images. The scratch size was measured at 0 and 18 h, and the result was reported as a bar chart.

Flow cytometric assays

The Annexin-V/PI assay and flow cytometry evaluated the MCF-7 and SKBR3 cells' apoptotic activation. Cells exposed to *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin were also compared. MCF-7 and SKBR3 cells were subjected to compositions at IC50 concentration for 24, 48, and 72 h to assess the apoptosis ratio. The cells were then analyzed using an Annexin V/propidium iodide (PI) assay according to the manufacturer's guidelines. Cells were re-suspended in 1X binding buffer after two washes with cold PBS (5×10^5 cells/well). The solution was transferred into a 5 mL culture tube at a volume of 100 µl. Five microliters of FITC Annexin V and propidium iodide were added to the tubes. A gentle vortex was recommended, followed by a 15-min incubation in the darkroom at 25 °C. The tubes were filled with 400 µl of 1X binding buffer. Flow cytometry was employed for analysis. The untreated cells served as a control.

Cell cycle analysis

Absolute ethanol was used to fix the cells for 24 h. The cells were washed twice in PBS before being stained for 15 min employing BD Bioscience Pharmingen's PI/RNase staining reagent. Flow cytometry was used to determine the DNA content of the cell population. FlowJo V10 software (Tree Star, Ashland, OR) evaluated the cell cycle data.

Transcription of apoptosis-related genes by quantitative real-time PCR

The transcription of the antiapoptotic genes *BCL2*, *P57*, *Caspase3*, *Caspase9*, *Bax*, *MMP2* and *MMP9*, was measured using a quantitative real-time PCR technique with

SYBR green detection. The manufacturer's guidelines used the RNX TM-PLUS solution (Sinaclon, Iran) to extract Total RNA. Subsequently, cDNA was synthesized employing the AmitisGen Tech Dev group Manufacturers Kit (AmitisGen, Iran). According to the manufacturer's guidelines, quantitative real-time PCR was conducted using a SYBR® Premix Ex TaqTM II kit (TaKaRa, Japan) and specific primers (Table 2). Relative gene transcription levels were determined by normalizing each gene's expression to the *GAPDH* level.

Statistical analysis

The data were analyzed, and statistical analyses were conducted employing GraphPad Prism 5.0. The supplied data were mean and standard deviation. A one-way analysis of variance (ANOVA) was used to compare means, followed by a Tukey–Kramer post hoc test with a 95% confidence interval. A chi-square analysis with Yates' correction was conducted to assess the survival rates between vaccinated animals and the control group. Differences were considered significant and highly significant at $P<0.05$ and $P<0.01$, respectively.

Results

Generating and identifying recombinant pDNA/*Melittin*

The pDNA/*Melittin* vector was created by inserting the recombinant *Melittin* gene into the expression plasmid pDNA. The genetic content of the recombinant plasmid was utilized to assess the efficacy of the cloning procedure. The whole sequence of the recombinant plasmid is shown in Supplementary Table S1. Furthermore, DNA sequencing demonstrated that the sequence of the recombinant plasmid exhibited similarity to that of bee venom *Melittin*. BamHI and EcoRV were utilized to cleave the synthesized plasmid. The successful production of the recombinant plasmid was

Table 2 List of specific primers used in this research

Gene	Primer	Sequence (5′–>3′)	GenBank	TM(°C)
P57	*P57*-F *P57*-R	CTCCAGCTCCTTCCTCCTCT GAGACGCGCACATCTTCATA	D44497.1	60
BCL2	*BCL2*-F *BCL2*-R	AAGGGGGAAACACCAGAATC ATCCTTCCCAGAGGAAAAGC	KY098799.1	60
Caspase3	Cas3-F Cas3-R	TTTTTCAGAGGGGATCGTTG CGGTTAACCCGGGTAAGAAT	NM_001354783.2	60
Caspase9	Cas9-F Cas9-R	CTAGTTTGCCCACACCCAGT GCATTAGCGACCCTAAGCAG	NM_032996.3	60
Bax	*Bax*-F *Bax*-R	GACGGCCTCCTCTCCTACTT CCTCCCAGAAAAATGCCATA	NM_001291431.2	60
MMP2	*MMP2*-F *MMP2*-R	ACAGCAGGTCTCAGCCTCAT TGAAGCCAAGCGGTCTAAGT	NM_001127891.3	60
MMP9	*MMP9*-F *MMP9*-R	TTGACAGCGACAAGAAGTGG GCCATTCACGTCGTCCTTAT	NM_004994.3	60
GAPDH	GDH-F GDH-R	CAACGAATTTGGCTACAGCA AGGGGTCTACATGGCAACTG	NM_001256799.3	60
Melittin	Mel-F Mel-R	ATGGGAATTGGAGCTGTGC CTGCTGTCTCTTTCTCTTGATC	–	60

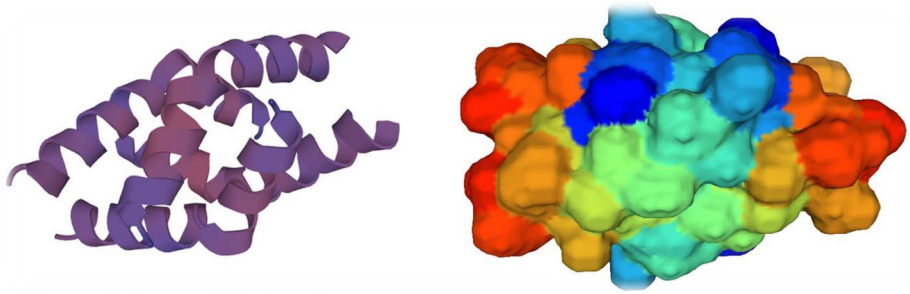

Fig. 1 Three-dimensional structure of the *Melittin* peptide

Table 3 Morphological features of recombinant pDNA/*Melittin*/pectin NPs

Formulations	Polydispersity index	Zeta potential (m.v)	DLS	TEM (nm)	EE (%)
Pectin	0.167 ± 0.25	4 ± 0.33	219 ± 3.12	145 ± 2.54	–
pDNA/*Melittin*/pectin NPs	0.207 ± 0.43	8 ± 0.56	238 ± 2.73	193 ± 3.42	81.43 ± 1.43

Average ± SD was use to analysis

confirmed by electrophoretic separation of the digestion fragments at 81 bp, corresponding to the *Melittin* gene. The 3D structure of the *Melittin* peptide is shown in Fig. 1.

Features of *Melittin*-loaded pectin (pDNA/*Melittin*/pectin)

An effective drug delivery system requires high encapsulation effectiveness, compact dimensions, and a structure capable of transporting sufficient medicines to the target area for particle release. The link between size and encapsulation efficacy in pectinal formulations must be clarified. Pectinal formulations have been developed and studied to enhance the arrangement. Table 3 presents the pectinal formulation's dimensions, polydispersity index (PDI), and encapsulation efficiency (EE). Transmission electron microscopy (TEM) methods evaluated the morphology and dimensions of the nanoparticles. The SEM and TEM analysis (Fig. 2A) revealed that the pDNA/*Melittin*/pectin nanoparticles exhibited uniformity and consistently spherical morphology. The diameters of free pectin and pDNA/*Melittin*/pectin nanoparticles were measured using particle size analysis, yielding values of 145 ± 2.54 nm and 193 ± 3.42 nm, respectively. Both measurements fall within the nano-range (100–200 nm) and exhibit satisfactory uniformity, indicated by a polydispersity index (PDI) ranging from around 0.167 ± 0.25 to 0.207 ± 0.43. The dimensions of nanoparticles measured by DLS for free pectin and pDNA/*Melittin*/pectin NPs were 219 ± 3.12 nm and 238 ± 2.73 nm, respectively (Fig. 2B). This disparity may arise from the procedural variations between SEM and DLS; SEM examines dry materials, but DLS analyzes potentially moist samples, leading to a more pronounced particle size in the DLS results. The research demonstrates a consistently distributed smooth surface, a spherical configuration, and a delineation from boundaries. The zeta potentials for free pectin and pDNA/*Melittin*/pectin nanoparticles were 4 ± 0.33 mV and 8 ± 0.56 mV, respectively, as shown in Table 3.

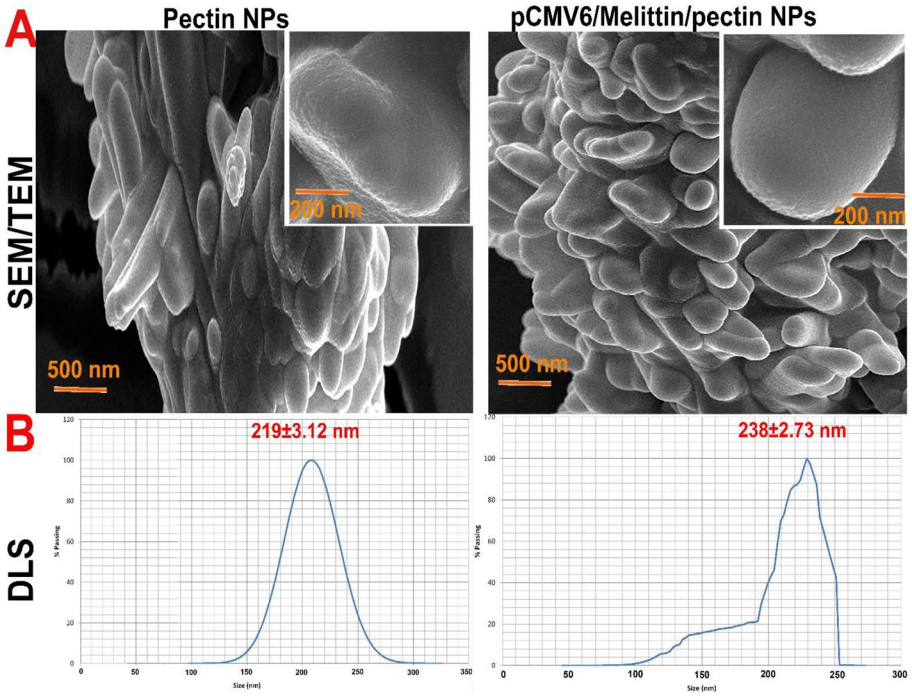

Fig. 2 Morphological features of pectinal formulations compared to blank pectin. **A** SEM and TEM image of nanoparticles. **B** DLS analysis of formulations

The efficiency of *Melittin* loading rate

The differential fluorescence of the OliGreen nucleic acid indicator shows the quantity of encapsulation of the recombinant plasmid used in the experiment. The fluorescence peaks when a sample includes a free plasmid. The absence of fluorescence indicates that the recombinant plasmid has grown too complex to bind DNA dyes and generate fluorescence effectively. The absorbance spectra of the recombinant plasmid show an RFU drop of 85.34 ($P < 0.01$). The ideal ratio for preserving and transmitting the whole DNA load is achieved after the complete digestion of the recombinant plasmid (Fig. 3A).

Studies of the Pectinal *Melittin* released in vitro

The drug release pattern of each selected formulation was monitored for 72 h at body temperature and pH values of 7 and 5 to comprehend the in vitro drug release better. The free medicines first entered circulation, as seen in the "Release" graph (Fig. 3B), and after 24 h, they exhibited a consistent release pattern in the subsequent hours. Based on the pectin release pattern, over 50% of the pectin is released during the first 8 h. At pH 5, the release is 82%, but at pH 7, it is just 57%. In addition, the release rate is 97% in acidic circumstances (pH 5) and 77% at pH 7 after 72 h (Fig. 3B). Surveillance of the release pattern of pectinal *Melittin* indicated that 34% of the medicine penetrated during the first 8 h at pH 7. However, this rate increased to 57% at pH 5. Surveillance of pectinal *Melittin* release patterns after 72 h indicated that the acidic environment, which induced swelling of the pectineus structure, resulted in the release of 53% and 73% of the drug into circulation at pH 7 and pH 5, respectively (Fig. 3B). The acidic release of pectin is associated with electrophilic addition mechanisms. The release rate of the

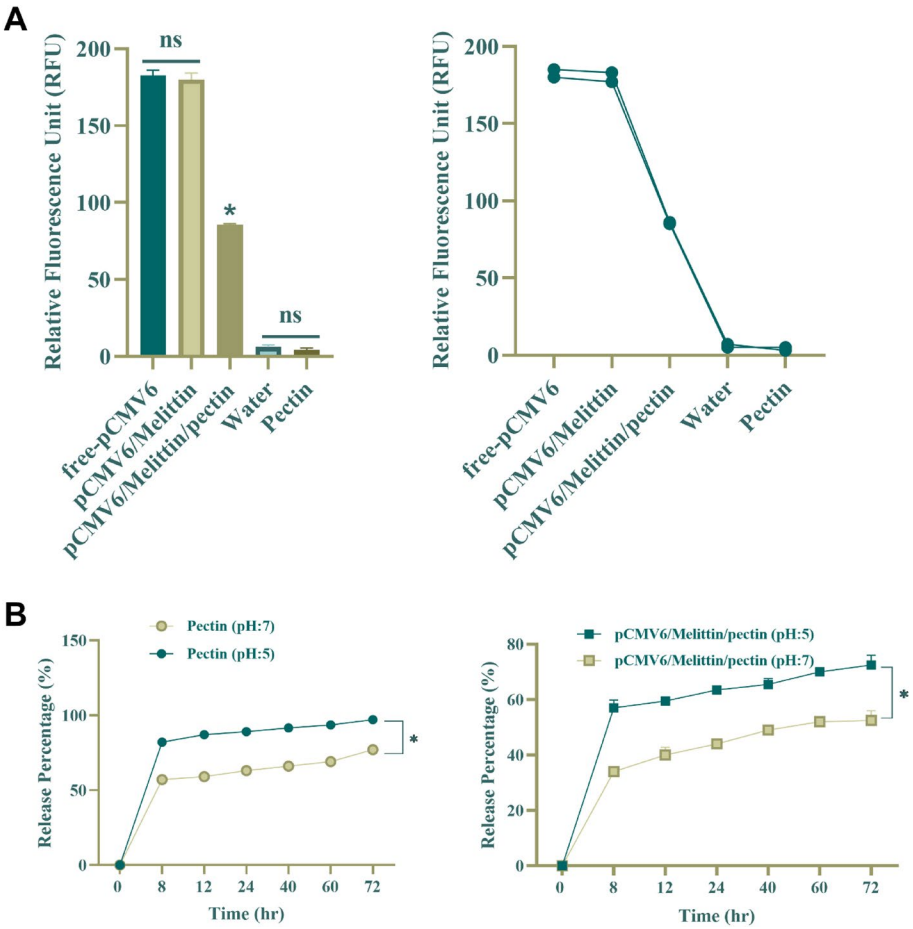

Fig. 3 Pectin NPs and Pectinal *Melittin* realized pattern. **A** Quantity of Pectinal *Melittin* NPs determines the relative fluorescence of recombinant plasmid in each contact. Most fluorescence is found in free recombinant plasmids. The Pectinal *Melittin* NPs exhibit a reduction in fluorescence intensity. **B** Comparison of the in vitro drug release profiles of pectin and pectinal *Melittin* NPs from the dialysis tube at different pHs (7 and 5) at 37 °C. Evaluating the optimal formulation's stability between 4 and 25 °C

pectin-loaded medication has been examined. The acidic environment of tumor wards compromised the pectineus structure, increasing the frequency of release and toxicity. Furthermore, the acidic milieu influences *Melittin* and elevates osmotic pressure, increasing cytotoxicity.

Pectinal *Melittin* physical stability

To determine the optimal *Melittin* pectinal formulations and their physical stability, particle size, polydispersity index (PDI), and encapsulation efficiency (EE) were assessed at 4 °C and 25 °C on days 0, 15, 30, 45, and 60 post-productions. The results indicated that temperature did not influence particle size, PDI, or EE on the first day. The pDNA/*Melittin*/pectin formulation exhibited the lowest size, averaging 193 ± 3.42 nm, the greatest PDI at 0.207 ± 0.43, and the highest EE at $81.43 \pm 1.43\%$ at 4 °C. The size of the pDNA/*Melittin*/pectin at 4 °C was 193 nm at zero and 211 nm after 60 days. In contrast, the pDNA/*Melittin*/pectin at 25 °C measured 193 nm at zero and 513 nm after 60 days (Fig. 4A, D). The pDNA/*Melittin*/pectin stored at

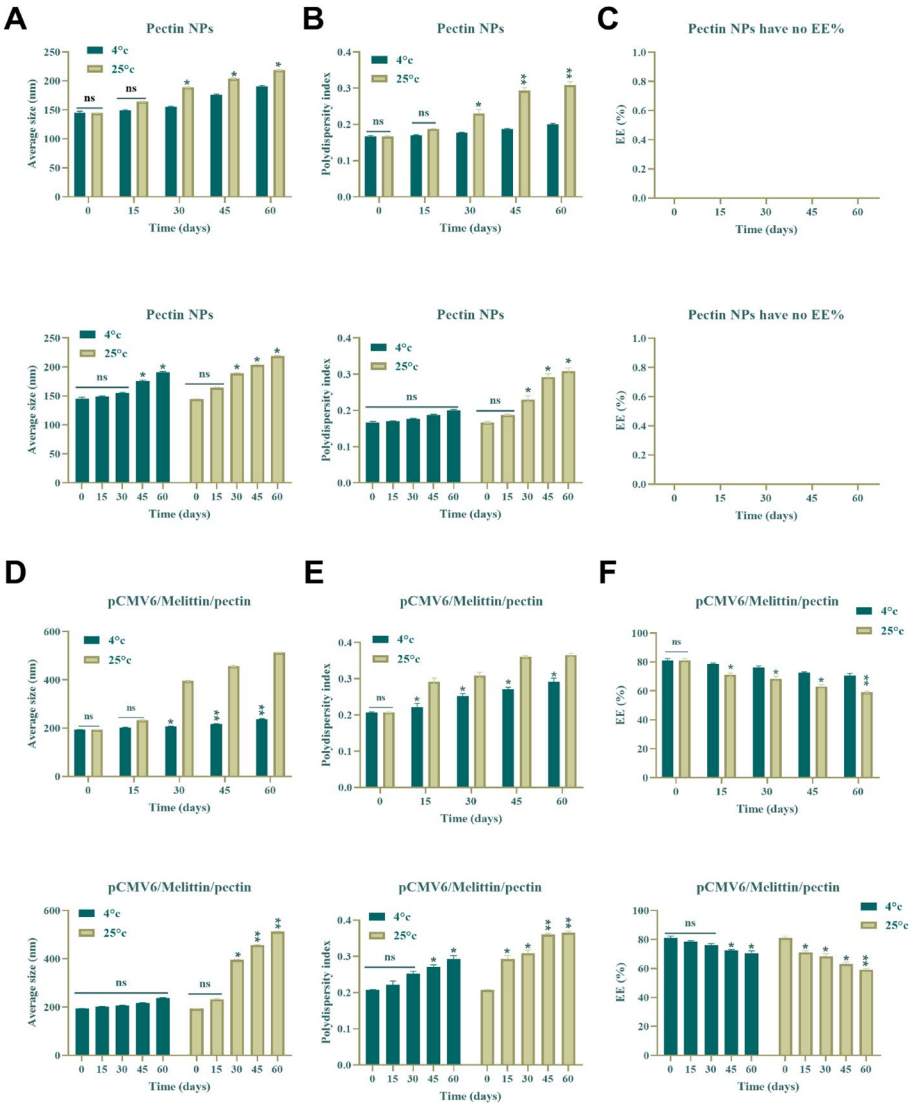

Fig. 4 Pectin NPs and Pectinal *Melittin* physical stability pattern. Three stability factors were investigated: average particle size (**A**), PDI (**B**), and EE% (**C**) of pectin NPs and average particle size (**D**), PDI (**E**), and EE% (**F**) of pDNA/*Melittin*/pectin. The results are shown as mean ± SD (n = 3). *P < 0.05, **P < 0.01

4 °C exhibited a polydispersity index (PDI) of 0.207 at day 0 and 0.292 at day 60. The pDNA/*Melittin*/pectin at 25 °C exhibited a polydispersity index (PDI) of 0.207 at day 0 and 0.365 at day 60. The pectin at 4 °C exhibited a polydispersity index (PDI) of 0.167 at 0 days and 0.200 at 60 days. The pectin exhibited a polydispersity index (PDI) of 0.167 at 25 °C and 0.308 at zero and 60 days (Fig. 4B, E). The pDNA/*Melittin*/pectin at 4 °C exhibited an encapsulation efficiency (EE) of 81.43% at day 0 and 70.56% at day 60. The pDNA/*Melittin*/pectin exhibited an encapsulation efficiency (EE) of 81.43% at 0 days and 59.06% at 60 days when maintained at 25 °C (Fig. 4C, F). The findings demonstrate that stability is better at 4 °C than 25 °C, where enlarged pores led to increased size, elevated PDI, and reduced EE. This is due to the rigidity and pliability of the pectins.

pDNA/*Melittin*/pectin NPs suppress malignant cell proliferation in vitro

A dose–response investigation was conducted using MTT to evaluate the cytotoxicity in different groups. The proliferation of MCF-7 and SKBR3 cells was suppressed by free *Melittin* and pDNA/*Melittin*/pectin in a dose-dependent pattern. In all study groups, including the MCF-7 cell line, the IC50 was evaluated. The IC50 values for *Melittin*, Pectin, pDNA/*Melittin*, and pDNA/*Melittin*/Pectin in the study groups were 40 µg/mL, 80 µg/mL, 10 µg/mL, and 2.5 µg/mL, respectively, after 24 h. This indicates that the toxicity of *Melittin* against cancer cells is amplified through its nano-encapsulation within pectin (Fig. 5B). The concentration of IC50 in the SKBR3 cell was measured following a 24-h treatment period. The IC50 values were 20 µg/mL, 80 µg/mL, 10 µg/mL, and 2.5 µg/mL for the *Melittin*, Pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin groups, respectively (Fig. 5A). The MCF10A human breast standard cell line was treated for 24 h. The concentrations of IC50 for *Melittin*, pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin were 5 µg/mL, 80 µg/mL, 10 µg/mL, and 80 µg/mL. Figure 5B shows that the formulation of nanopectin is responsible for the specificity of pDNA/*Melittin* administration to cancer cells. It has been shown that acidic conditions increase the rate at which pectinal *Melittin* is released. pDNA/*Melittin*/pectin exhibits greater selectivity toward cancer cells due to their acidic microenvironments.

The MTT experiment revealed that empty pectin dilutions had no significant cytotoxic effect on MCF10A cells. The cell viability percentages of different formulation dilutions on the MCF10A, SKBR3, and MCF-7 cells were 100%. The cell viability percentages of

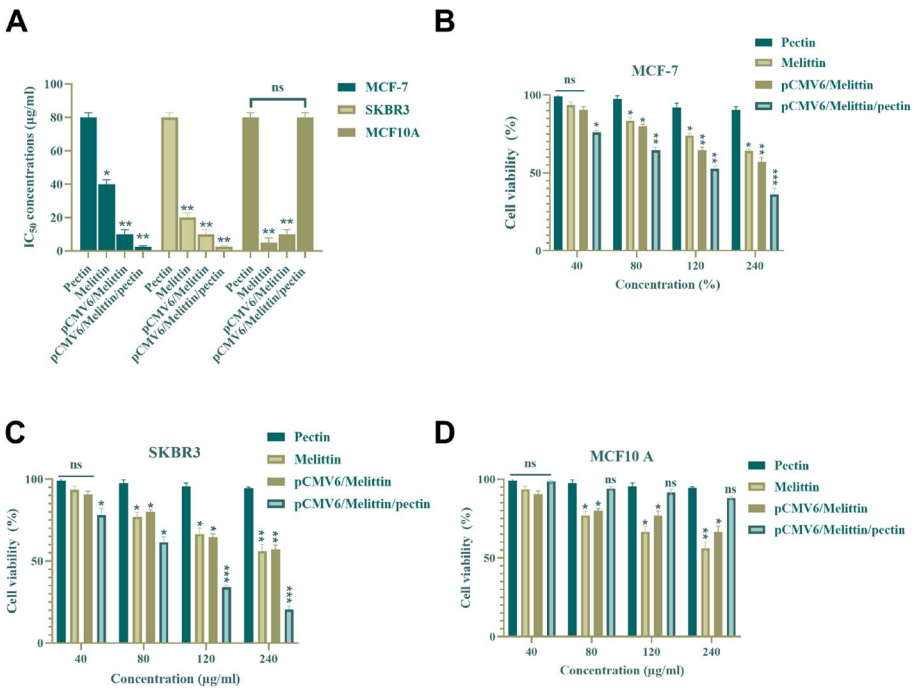

Fig. 5 **A** IC50 values in the study groups after 24-h treatment of the MCF-7, SKBR3, and MCF-10A cell lines with *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin were statistically significant ($P < 0.001$***). The impact of dilute solutions of *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin on cell viability were assessed in MCF-7 (**B**), SKBR3 (**C**), and MCF-10A (**D**) cell lines. Data is presented as mean ± SEM

different formulation dilutions on MCF-7 (Fig. 5B), SKBR3 (Fig. 5C), MCF10A (Fig. 5D) cell lines were documented.

Wound healing analysis

The MCF-7 and SKBR3 cell lines administered pDNA/*Melittin*/pectin showed reduced cell migration compared to Free *Melittin*. A statistically significant enhancement in scratch width was observed in SKBR3 cells administered pDNA/*Melittin*/pectin (95.56 µm) relative to pDNA/*Melittin* (84.32 µm), *Melittin* (46.24 µm), and pectin (22.25 µm) (Fig. 6A). In addition, MCF-7 cells administered pDNA/*Melittin*/pectin showed a statistically significant increase in scratch width (97.42 µm) in comparison with pDNA/*Melittin* (65.23 µm), *Melittin* (33.41 µm), and pectin (17.24 µm) (Fig. 6B). In MCF-7 cells administered pDNA/*Melittin*/pectin, the scratch width

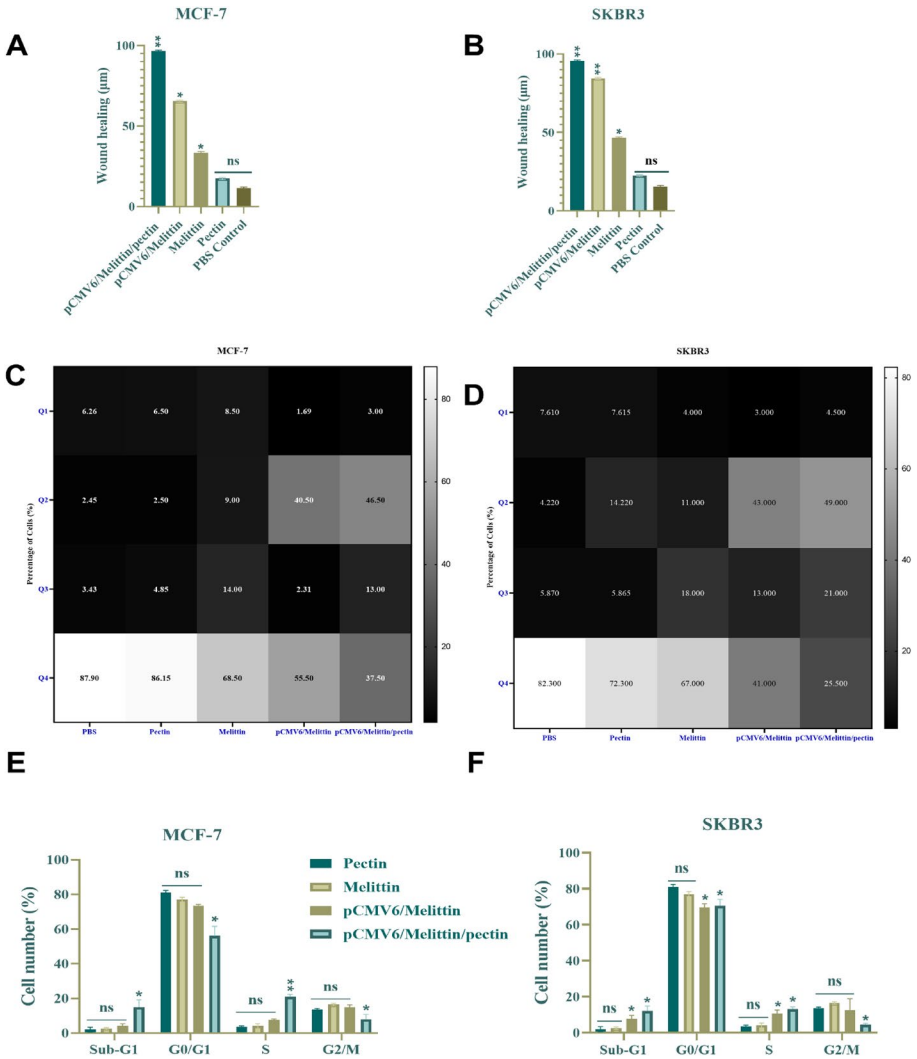

Fig. 6 Gap contour in the pDNA/*Melittin*/pectin group was higher than other groups (after 18h) in MCF-7 (**A**) and SKBR3 (**B**). Flow cytometric investigation diagrams were shown in MCF-7 (**C**) and SKBR3 (**D**) breast cancer cell lines. The cell cycle was regulated in MCF-7 (**E**) and SKBR3 (**F**) breast cancer cell lines

increased statistically considerably ($P < 0.01^{**}$) compared to *Melittin* (39.57 μm). In SKBR3 and MCF-7 cells treated with PBS, the scratch sizes measured 15.36 μm and 11.37 μm, respectively.

Flow cytometric analysis

Flow cytometric study diagrams for *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin were shown in the SKBR3 and MCF-7 breast cancer cell lines, respectively. The results indicated that the simultaneous administration of *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin enhanced overall apoptosis in both the MCF-7 and SKBR3 cancer cell lines (Fig. 6C, D). The data indicate no significant changes in the apoptosis rate (%) among the groups administered blank pectin and the control group in either cell line. PDNA/*Melittin* and pDNA/*Melittin*/pectin exhibited a statistically significant increase in apoptosis frequency compared to controls in both cell lines administered *Melittin* ($P < 0.001^{***}$). Compared to *Melittin*, pDNA/*Melittin* and pDNA/*Melittin*/pectin exhibited a higher apoptotic rate (%) in both cell lines.

Flow cytometry was used to analyze the cell cycle regulation in cell groups including *Melittin*, blank pectin, pDNA/*Melittin*, and pDNA/*Melittin*/pectin. PDNA/*Melittin*/pectin cells exhibited elevated G0.G1 ratios and reduced S phase to G2.M phase ratios compared to the control groups. The findings indicated that pDNA/*Melittin*/pectin inhibited cell cycle progression (Fig. 6E, F).

pDNA/*Melittin*/pectin increased apoptosis rate using suppress apoptosis-related genes

The transcription levels of the proapoptotic genes *P57, Caspase3, Caspase9,* and *Bax*, with the antiapoptotic genes *BCL2, MMP2*, and *MMP9*, were assessed utilizing real-time PCR in two cell lines. The pDNA/*Melittin*/pectin groups dramatically increased proapoptotic gene transcription compared to the other groups Subsequently, we examined the antiapoptotic genes *BCL2, MMP2*, and *MMP9* transcription in two cell lines. The transcription of *BCL2, MMP2*, and *MMP9* genes was elevated in the control cell line compared to the pDNA/*Melittin*/pectin groups ($P < 0.05$). Figure 7 illustrates that *BCL2*, *MMP2*, and *MMP9* transcription levels in both cell lines across all experimental groups were significantly lower than in the control group ($P < 0.01^{**}$). In contrast*, Caspase3, Caspase9*, and *Bax* transcription levels were markedly elevated in both cell lines across all experimental groups.

Melittin exhibited reduced *caspase9* transcription levels compared to the pDNA/*Melittin*/pectin group ($P < 0.05^{*}$). Compared to the control group, the pDNA/*Melittin* exhibited a statistically significant increase in *caspase9* transcription level ($P < 0.05^{*}$). Nonetheless, pDNA/*Melittin*/pectin exhibits higher *Bax* transcription levels in cells compared to *Melittin* ($P < 0.05^{*}$). While compared to the PBS group, there was a significant increase in level $P < 0.01^{**}$. SKBR3-related data indicate that pDNA/*Melittin*/pectin has reduced gene transcription levels of *MMP2* and *MMP9* compared to *Melittin* and pDNA/*Melittin* ($P < 0.05^{*}$). While compared to the PBS group, there was a significant decrease in level $P < 0.01^{**}$ (Figs. 8 and Fig. 9).

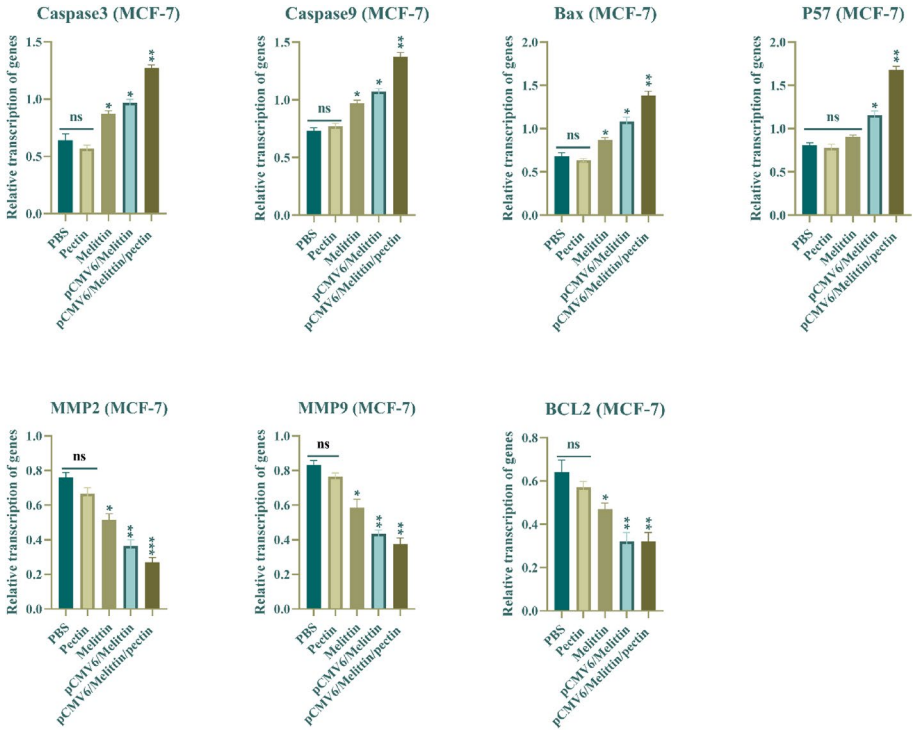

Fig. 7 Pectinal *Melittin* leads to induction of transcription of pro-apoptotic genes *P57, Caspase3, Caspase9,* and *Bax* at a significant level **P < 0.01 and reduction of expression of anti-apoptotic genes *BCL2, MMP2,* and *MMP9* at a significant level **P < 0.01 in the MCF-7 cells. Data were normalized by the *GAPDH* reference gene. There was no significant difference in the transcription of pro/anti-apoptotic genes in the Blank control groups

Discussion

Melittin is composed of residues of amino acids. Because of its absence of cell selectivity, it has been shown to have strong hemolytic activity on both microbial and human cells (Piri Gharaghie et al. 2021; Memariani et al. 2019).

Non-specific cellular lytic activity and rapid blood dissolution are two complicated issues linked to Melittin's characteristics (Askari et al. 2021), which might be troublesome. Intravenous administration of Melittin elicits significant adverse reactions, including hemolysis, which restrict its widespread use in cancer therapy (Piri Gharaghie et al. 2021; Askari et al. 2021). Since pharmacological research indicates that the biological environment's negative effects on the medicinal product are one of the main reasons drug deliveries to the target cell are delayed, we employed pectin as a Melittin nanocarrier in this study because of its structural advantages. Pectin shields the drug (Kapoor et al. 2024), has a high level of stability, and has a longer shelf life (Khotimchenko et al. 2020). The controlled-release nano-system improves drug residence and durability in the targeted organ for prolonged periods, and the nanocarrier has a high cell level of absorption (Ameta et al. 2023). Pectin mitigates the drug's negative consequences, including cytotoxicity and hemolysis. Negative impacts and sharply increased breast cancer cells (Emran et al. 2022). P-glycoprotein (P-gp) is significantly reduced by pectins, enhancing the availability of anticancer and antiviral drugs (Gerber et al. 2018). The hemi-fusion technique enables tailored nano-pectins

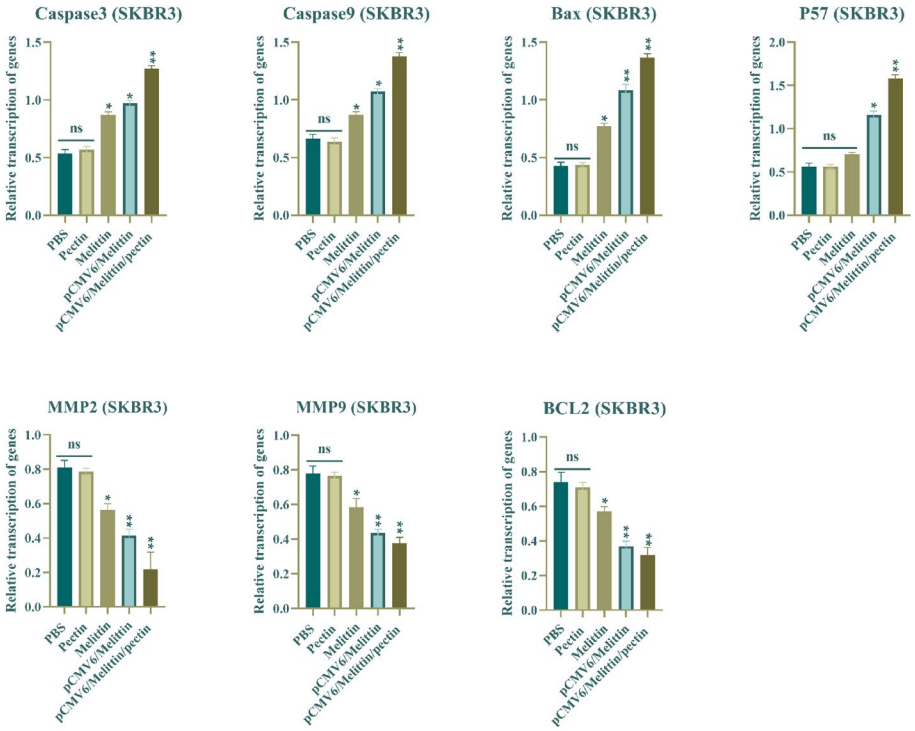

Fig. 8 Pectinal *Melittin* induces the transcription of pro-apoptotic genes *P57*, *Caspase3*, *Caspase9*, and *Bax* at a substantial level ($P < 0.01$) and reduces the transcription of anti-apoptotic genes *BCL2*, *MMP2*, and *MMP9* at a considerable level ($P < 0.001$). Data were standardized using the *GAPDH* reference gene. No substantial difference was seen in the transcription of pro- and anti-apoptotic genes in the Empty control groups

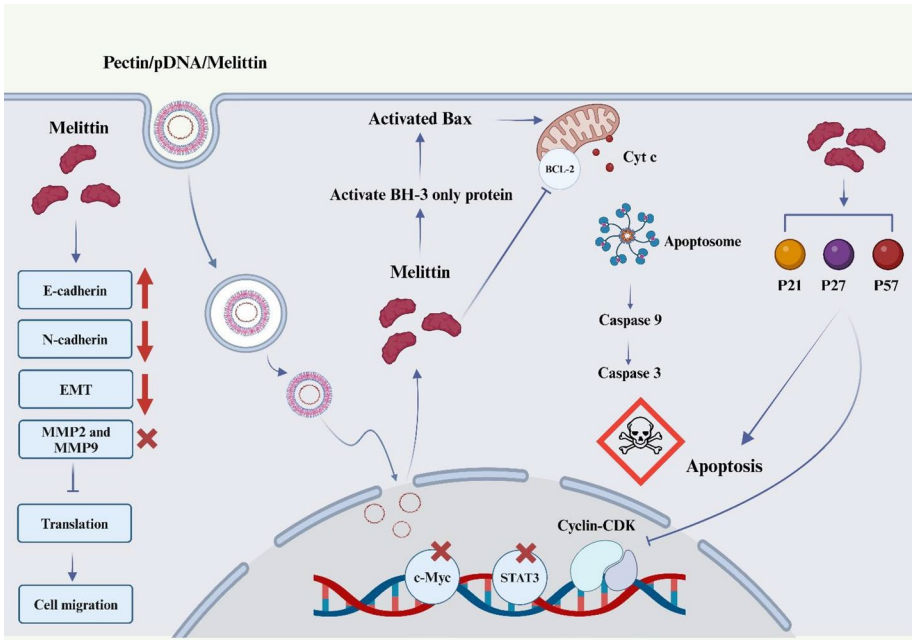

Fig. 9 Scheme illustrates biological efficacy and signaling inhibition of pCMV6/*Melittin*/pectin after cellular uptake

to deliver Melittin to malignant cells with excellent selectivity (Ganatra et al. 2022). In one study, Melittin-loaded nanoparticles unexpectedly exhibited more cytotoxicity than Melittin itself, along with superior stability and shelf life (Chary et al. 2024).

The study found that *Melittin*-loaded pectinal nanomaterials had no hemolytic effect on cancerous breast cells. Pectinal nanoparticles were used in the present study because of *Melittin*'s hemolytic effect. Nevertheless, the results indicate no hemolytic effect in this formulation relative to *Melittin*. The specifically engineered nanocarriers, including *Melittin*, inhibited the proliferation of malignant breast cell lines MDA-MB-231 and MCF-7, enhancing *Melittin*'s anti-cancer efficacy (Hussein et al. 2023). The cytotoxicity of *Melittin* against cancer cells was demonstrated in some studies (Gajski et al. 2013). *Melittin* may inhibit the calcium pump function of calmodulin, resulting in elevated intracellular calcium levels that are detrimental to cells (Jafari et al. 2024).The activation of L-type Ca2 + channels may elevate intracellular Ca2 + concentration, as demonstrated by many studies (Striessnig et al. 2014). *Melittin* influences intracellular Ca2 + mobilization and exocytosis via distinct signaling mechanisms (Ryu et al. 2014). Elevated intracellular calcium modulates the transcription of genes associated with apoptosis, notably enhancing the expression of the *Bax* gene and diminishing the expression of the *BCL2* gene, inducing cell death and promoting the up-regulation of the caspase 3 and 9 genes.

Melittin has been shown to have lethal effects on cells and to increase intracellular Ca2 + levels typically. However, its strong hemolytic activity is one of the most critical roles of *Melittin*, an arrangement of amino acids with the molecular composition C131H229N39O31 (Memariani et al. 2021). Most of the initial 20 amino acids that make up the *Melittin* molecule are hydrophobic. Nevertheless, most residues in the C-terminal domain are hydrophilic (Guha et al. 2021), facilitating this amphipathic configuration. Cellular phospholipid membranes might react favorably with *Melittin* (Guha et al. 2021). *Melittin* is a non-selective cytolytic peptide that tears down all bacterial and eukaryotic cell membranes (Mans et al. 2017). *Melittin* may have an efficient interaction with cellular phospholipid membranes (Guha et al. 2021; Mans et al. 2017).

The non-selective cytolytic peptide *Melittin* mechanically and chemically disrupts the membranes of all bacteria and eukaryotic cells. *Melittin* physically disrupts the cell membrane, significantly weakening the cell's solubility barrier through lysis (Guha et al. 2021; Mans et al. 2017). The peptide divides as a subunit into the cell membranes, where it oligomerizes to create toroidal or barrel-shaped stave formations, causing the thawing of apertures and resulting in cell mortality (Singer et al. 1972). *Melittin* activates executive caspases in many cells, notably U937, Hep3B, HepG2, and BEL-7402 (Guha et al. 2021; Mans et al. 2017; Singer et al. 1972). *Melittin* causes apoptosis in malignant cells by activating death receptors, enhancing the production of DR3, DR4, and DR6, and inhibiting the JAK2/STAT3 signaling pathway (Lyu et al. 2019). Separate research demonstrated that *Melittin* triggers apoptosis in cancerous cells by modulating the AKT signaling pathway, decreasing *BCL2* gene translation, increasing *Bax* gene expression, and enhancing the function of caspase 3 and 9 (Pandey et al. 2023). In addition, *Melittin* may induce apoptosis in the HCC cell line by activating JNK/p38 MAPK, which converts Ca2 + /calmodulin-dependent protein

kinase and growth factor-beta-activated kinase 1 (Wang et al. 2015). This study suggests *that melittin* may demonstrate its anticancer effects by generating initial apoptosis, reactive oxygen species activity, and caspase 3 functionality.

Moreover, *melittin* elevates cytochrome C and endoG while decreasing AIF by activating the mitochondrial mechanism. These modifications are essential for regulating increased *Bax* transcription, diminished *BCL2* transcription, and ultimately heightened caspase 3- and 9-mediated apoptosis (Wang et al. 2024). The results endorse the application of *Melittin* in anticancer therapy and provide evidence of its mechanisms by diminishing the invasion frequency of MCF-7 breast cancer cells and MMP-9 via the downregulation of cyclophilin A transcription (Jin et al. 2024). *Melittin* and Chrysin, present in honey bee venom, may eradicate chemo-resistant ovarian carcinoma cells via increasing *Caspase3* and *Caspase9* while down-regulating *BCL2* transcription (Dabbagh Moghaddam et al. 2021). The present study demonstrates that *Melittin* administration induces synergistic and enhanced upregulation of the genes *Bax*, *Caspase3*, and *Caspase9* and downregulation of the genes *BCL2*, *MMP2*, and *MMP9* in both types of cells.

Conclusion

The proposed research sought to provide more effective, more beneficial, and less detrimental therapy for breast cancer. Our investigation demonstrates that Melittin-loaded nano-pectin operates well under the ideal expected circumstances. The current work illustrates that pectins function as efficient vesicle transporters for the incorporation of medicines, namely, Melittin. In vivo and in vitro studies demonstrate that Melittin-encapsulated pectin has much superior anti-cancer activity compared to free Melittin. The industrialization of this composition is expected to decrease the incidence of breast cancer in the next years.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12645-025-00313-6.

Supplementary material 1.

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Author contributions
E-AB designed and performed experiments reported in the paper, analyzed data and wrote the manuscript; T-PG helped with experiments, data analysis, and contributed to manuscript editing; A-D and M-A help the internalization experiments, data analysis for internalization; A-AD and A-D provided the information of leukemia clinical treatments and current challenges.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Islamic Azad University of Shahrekord Branch in Iran (IR.IAU.SHK.REC.1400).

Competing interests
The authors declare no competing interests.

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