Journal of Drug Delivery Science and Technology 84 (2023) 104545

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Fabrication and characterization of bee venom-loaded nanoliposomes: Enhanced anticancer activity against different human cancer cell lines via the modulation of apoptosis-related genes

Alaa Abd El-Gawad [a], Mohamed A. Kenawy [a], Tamer M. El-Messery [b], Marwa E. Hassan [c], Aziza A. El-Nekeety [d], Mosaad A. Abdel-Wahhab [d,*]

[a] Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt
[b] International Research Centre "Biotechnologies of the Third Millennium", Faculty of Biotechnologies, ITMO University, Saint Petersburg, Russia
[c] Toxicology Department, Research Institute of Medical Entomology, Giza, Egypt
[d] Food Toxicology & Contaminants Department, National Research Centre, Dokki, Cairo, Egypt

ARTICLE INFO

Keywords:
Bee venom
Nanoliposomes
Anticancer activity
Cytotoxicity
Gene expression
Cell lines

ABSTRACT

Despite the advancement of cancer therapy, the disease ranks as the most important cause of global death. Bee venom (BV) exhibits different biological properties and showed *in vitro* cytotoxicity against different malignant cells. However, its application in humans still faces challenges due to its allergic reactions, pain at the administration sites, and other severe toxic reactions such as hemolysis nonspecific cytotoxic activity, tickling sensation, and anaphylaxis during clinical treatment. This study aimed to prepare and characterize BV-loaded nanoliposomes (BV@NLs) and evaluate their anticancer activity against HepG-2, MCF-7, and HCT-116 cell lines *in vitro*. The prepared BV@NLs were spherical with an average size of 230.9 ± 5.21 nm and a positive charge ζ-potential of 46.5 mV. BV@NLs showed strong anticancer activity against the three tested cell lines compared to crude BV. It showed selective cytotoxicity and was more effective against the HCT-116 cell line with IC_{50} of 4.16 μg/ml. BV@NLs also modulate the mRNA expression of the apoptosis-related genes. It could be concluded that the use of nanoliposomes as a drug delivery system for BV enhanced the anticancer activity *in vitro* against HepG-2, MCF-7, and HCT-116 cell lines.

1. Introduction

Bee venom (BV) is a complex mixture of bioactive peptides released by the honey bee (*Apis mellifera*) as a defensive weapon against the attack of various predators and invaders [1]. Melittin is the major biologically active component of BV; however, it also contains apamin, adolapin, degranulating peptide, and enzymes (phospholipase A2, and hyaluronidase) in addition to some nonpeptide ingredients such as histamine, norepinephrine, and dopamine [2,3]. The therapeutic use of BV and other honey bee products in traditional medicine date back several thousands of years since their medicinal activities were cited in religious books such as the Quran and the Bible [4,5]. BV is used for the treatment of various diseases [6,7] and exhibits different biological properties including *in vitro* cytotoxic activity against different malignant cells [8,9]. BV also exhibited a strong inhibition activity against mammary cell carcinoma proliferation [10,11]. It is effective in arthritis [12], pain, rheumatism, skin diseases [13,14], osteoarthritis and rheumatoid arthritis [15,16].

Despite the potential use of BV, especially melittin, against different types of cancer, its application in humans still faces challenges due to its nonspecific cytotoxic activity [17]. To avoid these difficulties, the application of nanoparticles-based delivery of BV may be an optimal solution to such problems. Thanks to nanotechnology, it was possible to invent effective test conjugates of BV against a diversity of human cancers in the preclinical models [18]. Several efficient and safe delivery systems have been developed for BV or its major constituent, melittin, and were able to reduce the hemolytic property and conserve the cytotoxic advantages of venom [19,20].

Cancer ranks as the most important cause of global death, causing more than 10 million deaths in 2020, or approximately one in six deaths [21,22]. In 2019, the WHO reported that cancer is the main reason for death and a serious barrier to increasing life expectancy in all countries

* Corresponding author.
E-mail addresses: mosaad_abdelwahhab@yahoo.com, ma.abdelwahab@nrc.sci.eg (M.A. Abdel-Wahhab).

https://doi.org/10.1016/j.jddst.2023.104545
Received 7 January 2023; Received in revised form 26 April 2023; Accepted 5 May 2023
Available online 6 May 2023

worldwide [23]. Cancer is the 1st and 2nd cause of death in people younger than 70 in 112 of 183 countries and it ranks 3rd and 4th in a further 23 countries [24]. Rectum, colon, prostate, lung, liver, and breast cancer are the most prevalent cancer types [25]. High body mass index, tobacco use, insufficient intake of vegetables and fruits, alcohol consumption, and insufficient physical activity are the most common reason for cancer [23].

Although the studies on the efficiency of drug delivery systems have grown up recently, some limitations including poor biodistribution, solubility, and rapid clearance at physiological pH besides the insufficient uptake by the cells which are still needed to be overridden. The applications of nanosystems have emerged along with the recent progress in cancer treatments to increase the concentration of drugs inside the cancerous cells and reduce their toxicity in healthy cells [26]. Additionally, the application of nanoparticles synthesized from different materials such as dendrimers, polymers, liposomes, carbon nanotubes, viruses, and metals (e,g. gold and iron oxide) are currently used to enhance the anticancer activities of the drugs and eliminate the systemic toxicity [27]. Liposome nanocapsules (NLs) particularly were used for several purposes in the cosmetic, food, and pharmaceutical industries [28]. Recently, liposome technology was used for the synthesis of several nutraceutical products e.g. enzymes, vitamins, and herbal extracts [29,30].

Liposomes are spherical vesicles consisting of one or more concentric lipid bilayers formed spontaneously by the dispersion of phospholipids in the aqueous solvents [31]. Liposomes are attractive drug delivery systems due to their biodegradability, biocompatibility, non-toxicity, non-immunogenicity, and the ability to act as targeted release-on-demand carrier systems for the water and oil-soluble bioactive constituents [32]. Liposomes may be prepared by using different steps of processing such as sonication, agitation, lyophilization, extrusion, thawing, and freezing, conventional methods include reverse phase evaporation, solvent injection, and detergent dialysis. However, the most widely used method is the hydration of the thin lipid membrane, known as the Bangham method, and is used to obtain vesicular-lipid forms [33,34]. Therefore, the aims of this study were: the preparation and characterization of nanoliposomes as a delivery system for BV and to evaluate the anticancer activity of BV-loaded nanoliposomes (BV@NLs) against different cancer cell lines.

2. Materials and methods

2.1. Materials

Chitosan (high molecular weight, Mw 165–175 kDa) was purchased from Sigma Aldrich (Paris, France). Soy lecithin (69.3% phosphatidylcholine, 9.8% phosphatidyl ethanol amine, and 2.1% lysophosphatidic choline) was provided by Lipoid AG (Ludwigshafen, Germany). Sodium acetate and glacial acetic acid were purchased from Carl Roth GmbH and Co., KG (Karlsruhe, Germany). Dried purified Bee venom (BV) of *Apis mellifera lamarckii* was purchased from Agricultural Research Center, Giza, Egypt.

2.2. Preparation of primary and secondary BV@NLs

Liposomes were prepared as described by Gültekin-Ozgüven et al. [35] with some modifications. For the preparation of primary liposomes, lecithin powder 2% (w/v) was dissolved in acetate buffer (pH 3.5; 0.1 M) overnight at room temperature. BV was dissolved in this lecithin solution at a ratio of 1:1 (w/v). Conventional liposome was prepared by homogenizing dispersions using a high shear disperser (DI-25 Yellow line, IKA) at 15000 rpm for 10 min then homogenized using ultrasonication (160 W power, 20 kHz frequency and with 50% pulse; Sonics, Vibra, Cell, USA) for 5 min. To obtain secondary liposomes, all liposome dispersions were added to chitosan solution (0.8%, w/w) dissolved in an acetate buffer solution with a ratio of 1:1 (w/w) and stirred overnight at room temperature.

2.3. Characterization of BV@NLs

2.3.1. Determination of zeta potential and particle size distribution

The zeta potential and particle size were determined by a dynamic light scattering instrument (Nano ZS, Malvern Instruments, and Worcestershire, UK). The refractive index for lecithin was 1.37 ± 0.02. The liposomal solutions were diluted to a concentration of 0.1% (w/w) before the measurement.

2.3.2. Fourier transforms infrared spectroscopy (FTIR)

The solution was initially freeze-dried using a laboratory freeze dryer (SUBLIMATOR, VaCo5, ZIRBUS technology, Germany), then the vibrational modes of different samples were studied using Bruker FT-IR tensor (Germany).

2.3.3. Transmission electron microscope (TEM)

Twenty μl of diluted samples were placed on a film-coated 200-mesh copper specimen grid for 10 min and the excess fluid was removed using filter paper. The grid was then stained with one drop of 3% phosphotungstic acid and allowed to dry for 3 min. The coated dried grid was examined under the TEM microscope (JEM-2100 Electron Microscope) and the samples were observed by operating 13 at 160 kV.

2.4. Cell lines

Hepatocellular carcinoma (HepG-2), mammary gland (MCF-7), and colorectal carcinoma (HCT-116) cell lines were obtained from the American Type Culture Collection (ATCC) via VACSERA Co. (Cairo, Egypt). Doxorubicin (DOX) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as a standard anticancer drug for comparison.

2.5. Cytotoxic activity of BV@NLs

2.5.1. Cell culture

The three cell lines mentioned above were used for determining the inhibitory effects of BV and BV@NLs on cell growth using the MTT assay [36]. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a 5% CO_2 incubator.

2.5.2. Cytotoxic activity of BV@NLs against cancer cell lines

The cell lines were seeded in a 96-well plate at a density of 1.0×10^4 cells/well at 37 °C for 48 h under 5% CO_2. After incubation, the untreated control cells and the cells that were treated with different concentrations of BV or BV@NLs (100, 50, 25, 12.5, 6.25, 3.125, 1.56 μg) and DOX (1 μM) as a positive control, were incubated for 24 h. After 24 h of treatment, 20 μl of MTT solution at 5 mg/ml was added and incubated for 4 h. A volume of 100 μl of DMSO (dimethyl sulfoxide) was added into each well to dissolve the purple formazan formed. The colorimetric assay was measured and recorded using a plate reader (EXL 800, USA) at an absorbance of 570 nm. The relative percentage of cell viability was calculated as (A_{570} of treated samples/A_{570} of the untreated sample) X 100. All the experiments were carried out in triplicates.

2.5.3. Gene expression analysis for apoptotic-related genes

The gene expression assay was carried out to evaluate the effect of BV and BV@NLs at the concentration of IC_{50} on the apoptotic-related genes of three tested cancer cell lines. DOX was used as control positive, DMSO was used as control negative, and all cells were incubated for 24 h before the extraction of RNA. In this assay, total RNA extraction, cDNA synthesis, and qRT-PCR were conducted on different cell lines treated with BV or BV@NLS at a concentration of IC_{50}, as previously described [37].

Briefly, RNA samples were isolated using TRIzol reagent RNA and the purity was assessed by the absorbance ratio at 260 nm and 280 nm. cDNA was prepared from samples of 1 μg of RNA with SuperScript II reverse transcriptase according to the manufacturer's protocol. Real-time PCR reactions were run on a Strata gene Mx3005P Real-Time PCR System (Agilent Technologies). SYBR green qPCR Master Mix kit was used for qPCR analyses. A 20-μl reaction volume using 1 μg of cDNA, 10 μM of forward and reverse primers, 10 μl TOPreal™qPCR 2 × PreMIX (SYBR Green with low ROX) (Enzynomics), and DNAse-free water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were chosen as housekeeping genes. The sequences of the primers utilized in this study are summarized in Table 1. Each specimen was repeated four times. Finally, the comparative delta-delta CT method ($2^{-\Delta\Delta Ct}$) was applied to compute the fold change [38].

2.6. Data analysis

Statistical analysis of the results was carried out by using the SPSS.11 program. The significance of the differences among treatment groups was determined by One Way- ANOVA followed by Duncan's multiple tests. The values were expressed as Mean ± SD and the data were considered to be significant if the probability had a value of $P < 0.05$.

3. Results

3.1. Characterization of BV@NLs

The results revealed that the average particle diameter of NLs was found approximately 230.9 ± 5.21 nm (Fig. 1A) and PDI was 0.201 ± 0.01 after the homogenization. The ζ-potential of liposomes was positively charged at about 46.5 ± 1.25 mV (Fig. 1B). The TEM micrograph of the BV@NLs (bee venom-loaded liposomes) showed a spherical shape (Fig. 1C, D, F).

The FT-IR spectra of BV@NLs (Fig. 2) were analyzed in their solid state to be unaffected by the strong water absorption. The IR spectra were recorded in the region from 4000 to 400 cm^{-1}, but the bands in the region 4000 - 1400 cm^{-1} were analyzed in detail since they are characteristic of OH groups while NH groups appeared at 3000–4000 cm^{-1} of different protonic species that undergo hydrogen bonding interaction. Another region of interest was the region from 1800 to 1400 cm^{-1}, characteristic of bending vibrations for the same group, as the bands in this region are wide and complex. In the lecithin IR spectra, the most intense bands are those corresponding to (i) the alkane bands corresponding to symmetric CH_2, the antisymmetric CH_2, the antisymmetric CH_3 stretching and the CH_2 scissoring vibrational modes at 3009, 2922, 2852 and 1464 cm^{-1}, respectively; (ii) the carbonyl stretching vibration, located at 1736 cm^{-1}, and (iii) the highly overlapped PO_2- and P–O–C infrared active vibrations in the region between 1228 and 924 cm^{-1}, centered around 1054 cm^{-1}. The FTIR spectrum of chitosan membranes without ionic crosslinking (Fig. 2), displays a broad band in the region of 3357 and 3301 cm^{-1} that is attributed to O–H and N–H stretching vibrations of functional groups acting in hydrogen bonds. Discrete bands were observed around 2867 cm^{-1} and attributed to C–H stretching vibrations. Also, they exhibited characteristic absorption bands at 1644 cm^{-1} (C═O stretching in amide group, amide I vibration), and at 1588 cm^{-1} (N–H bending in amide group, amide II vibration). For the BV spectrum, the observed peak at the absorbance region of 2920-2851 cm^{-1} indicates the free vibrations of N–H stretching. The FTIR spectrum of BV also showed the characteristic amide bands, i.e., amide I (1645 cm^{-1}), amide II (1537 cm^{-1}), and the bands at 1077 cm^{-1} that indicate unsystematic coilcon formation. The FTIR spectrum of chitosan/BV nano-conjugate revealed that the BV absorbance peak for N–H stretching at 2920 cm^{-1} was reduced and shifted to 3242 cm^{-1}(chitosan/BV). The amide I indicated band in the BV at 1645 cm^{-1} was also shifted to a lower wave number (1638 cm^{-1}) in BV/chitosan.

Table 1
Details giving primer sequences for the genes amplified.

List of genes	Primer sequences (5′–3′)	References	Product Length(bp)
GAPDH	5′-CAAGGTCATCCATGACAACTTTG-3′ 5′-GTCCACCACCCTGTTGCTGTAG-3′	[78]	496
β-actin	F: 5′-CCACCATGTACCCAGGCATT"-3′ R: 5′-CGGACTCATCGTACTCCTGC-3′	[79]	189
BAX	F: 5′-ATCCAGGATCGAGCAGGGCG-3′ R: 5′-GGTTCTGATCAGTTCCGGCA-3′	[80]	315
BCL-2	F: 5′-CACAAGAGGCCAAGGCTACCT–3′ R: 5′-CAGGAAAGCAGGAAGTCTCAA-3′	[81]	158
P53	5′-TTGCCGTCCCAAGCAATGGA-3′ 5′-TCTGGGAAGGGACAGAAGATG-3′	[82]	193

3.2. Cytotoxic activity

The cytotoxicity results of the tested BV or BV@NLs against the three cell lines (HepG-2, MCF-7, and HCT-16) compared to DOX (a standard anticancer drug) are given in Table 2. These results indicated that BV@NLs showed strong anticancer activity against the three tested cell lines ($p < 0.001$) although it was more effective against the HCT-116 cell line compared to HepG-2 and MCF-7. The recorded IC_{50} was 8.60, 8.21, and 4.16 μg/ml for HepG-2, MCF-7, and HCT-116, respectively. However, the IC_{50} for crude BV was 16.56, 15.50, and 16.20 μg/ml for HepG-2, MCF-7, and HCT-116, respectively, and the IC_{50} for DOX recorded 4.50, 4.17, and 5.23 μg/ml for the HepG-2, MCF-7, and HCT-116 cell lines, respectively.

3.3. Cell lines assay

3.3.1. HepG-2 cell lines

The effect of BV and BV@NLs on the cell line viability of HepG-2 showed that the percentage of viability of HepG-2 cell lines (Table 3) was 7.1% for BV@NLs, whereas it was 16.1% for BV and 6.3% for DOX as the control anticancer drug at a concentration of 100 μg for the three agents. The viability was increased significantly ($p < 0.05$) by the decrease in the drug concentration.

3.3.2. MCF-7 cell lines

The effect of BV and BV@NLs on the viability percentage of MCF-7 cell lines is presented in Table 4. These results showed that both BV and BV@NLs affect negatively the viability of MCF-7 and the percentage reached 16.1 and 8.4% for BV and BV@NLs, respectively compared to the DOX which recorded 6.2% at a concentration of 100 μg. The viability was increased significantly ($p < 0.05$) by decreasing the drug concentration, and no cell death was observed at the low concentration (15.6 μg).

3.3.3. HCT-116 cell line

The results indicated that both BV@NLs were also effective against the HCT-116 cell line (Table 5). The cell viability at the concentration of 100 μg of DOX, BV, or BV@NLs was recorded at 7.1, 13.1, and 9.5%, respectively. The cell viability was increased significantly ($p < 0.001$) by the increase in the concentration of drugs and the lowest concentration (3.125 μg) did not show any response and the percentage of cell viability was recorded at 100%.

3.3.4. Apoptosis-related gene expression

The gene expression results showed that treatment with BV increased the Bax mRNA expression (Fig. 3A) in the tested cell lines and this increase reached 1.7, 1.69, and 1.72 folds in HepG-2, MCF-7, and HCT-

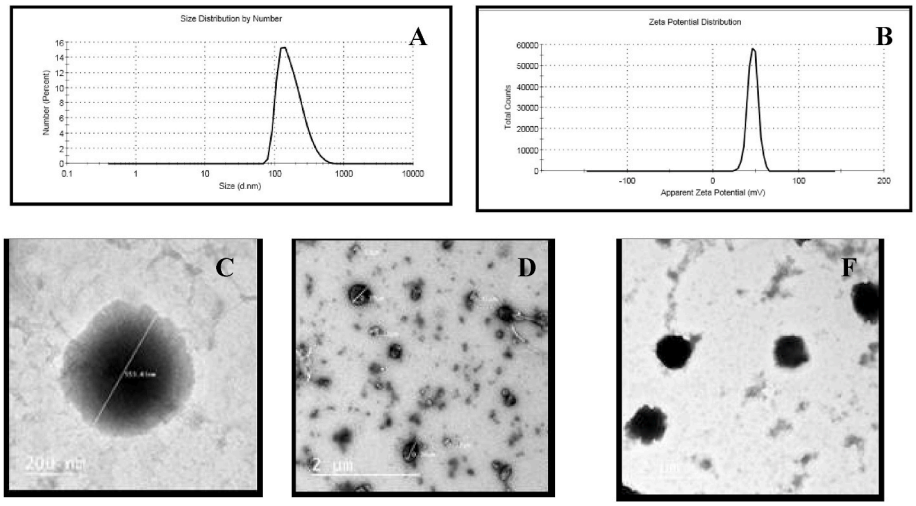

Fig. 1. Size distribution of liposomes (A), Zeta potential distribution (B), and TEM micrograph (C, D, F) of BV@LPs with 1% obtained at a scale of 0.2 μm (200 nm).

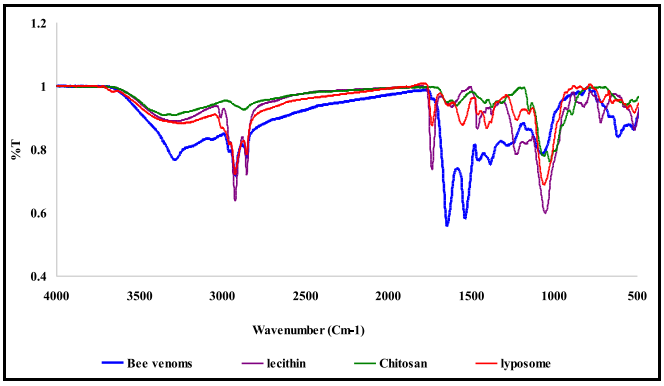

Fig. 2. FTIR Spectrum for Chitosan/bee venoms/lecithin nanoparticles.

Table 2
Cytotoxic activity of BV and BV@NLs against human cancer cell lines.

Compound	*In vitro* Cytotoxicity IC_{50} (μg/ml)[a]		
	HepG-2	MCF-7	HCT-116
DOX	4.50 ± 0.2	4.17 ± 0.2	5.23 ± 0.3
BV	16.56 ± 2.8	15.50 ± 1.9	16.20 ± 3.4
BV@NLs	8.60 ± 2.3	8.21 ± 2.1	4.16 ± 2.5

DOX: Doxorubicin; BV: Bee Venom; BV@NLs: Bee Venom loaded liposome.
[a] IC_{50} (μg/ml): 1–10 (very strong). 11–20 (strong). 21–50 (moderate) [83]. 51–100 (weak) and above 100 (non-cytotoxic).

Table 3
The average percentage of relative viability of HepG-2 cells line.

Conc. (μg)	DOX (Control)	BV	BV@NLs
100	6.3	16.1	7.1
50	11.2	25.7	12.6
25	14.1	34.8	15.8
12.5	28.3	40.9	25.7
6.25	45.8	65.2	40.2
3.125	57.6	92	67.3
1.56	71.2	100	100

116 cell lines, respectively. However, treatment with BV@NLs showed more increase in the mRNA expression of Bax reaching 2.55, 2.76, and 2.49 folds in HepG-2, MCF-7, and HCT-116 cell lines, respectively. The effect of BV or BV@NLs on Bcl-2 mRNA expression (Fig. 3B) revealed

Table 4
The average percentage of relative viability of MCF-7 cells lines.

Conc. (μg)	DOX (Control)	BV	BV@NLs
100	6.2	16.1	8.4
50	10.9	24.2	17.2
25	14.3	49.7	28.1
12.5	26.9	62.0	32.6
6.25	41.5	74.8	53.7
3.125	58.4	95.3	78.3
1.56	69.1	100	100

Table 5
The average percentage of relative viability of HCT-116 cells.

Conc.(μg)	DOX (control)	BV	BV@NLs
100	7.1	13.1	9.5
50	13.9	25.7	12.8
25	18.7	69.3	29.1
12.5	31.4	78.2	40.9
6.25	47.9	93.6	65.6
3.125	60.5	100	100
1.56	73.8	100	100

that both treatments induced a significant decrease ($p < 0.05$) in the expression of this gene. Treatment with BV reduced the Bcl-2 expression by 0.78, 0.77, and 0.86 folds; meanwhile, BV@NLs reduced the expression of Bcl-2 by 0.55, 0.64, and 0.54 folds in HepG-2, MCF-7, and HCT-116 cell lines, respectively. Moreover, the expression of p53 mRNA (Fig. 3C) was decreased by 0.84, 0.84, and 0.86 fold; however further decrease was observed in the cell lines treated with BV@NLs since it reached 0.42, 0.43, and 0.43fold in HepG-2, MCF-7, and HCT-116 cell lines, respectively.

4. Discussion

Bee venom (BV), particularly honey bee venom is well known to contain a diversity of biologically active proteins, including toxins that make it a potent drug widely used in pharmacology. The therapeutic application of BV has been used in traditional medicine for the treatment of several inflammatory diseases [39,40] such as rheumatoid arthritis or multiple sclerosis [41]. BV also exhibited cytotoxic activity against malignant cells [12], inhibited the proliferation of mammary cell carcinoma [11], and for the treatment of different types of cancer [42,43]. However, the clinical application of BV in cancer treatment faces several limitations due to the serious side effects such as allergic reactions, pain

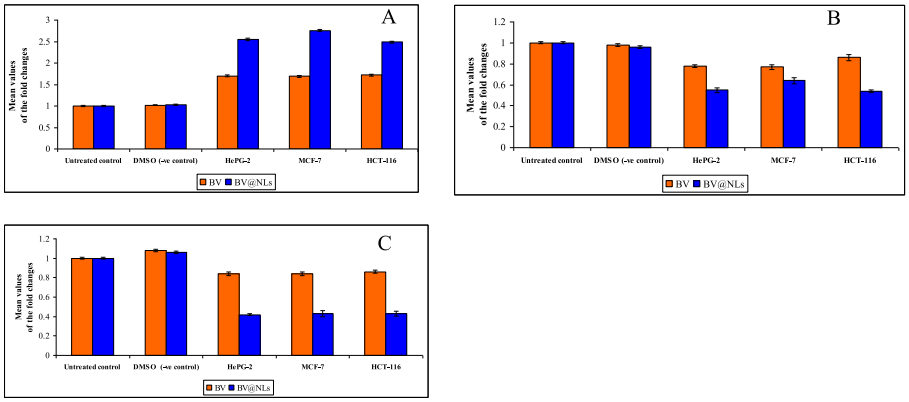

Fig. 3. The expression level of apoptosis-related gene **(A)** BAX, **(B)** Bcl-2 and **(C)** P53 in HepG-2, MCF-7 and HCT-116 cell lines treated with BV or BV@NLs at a concentration of IC_{50} presented in Table 2.

at the administration sites, and other severe toxic reactions such as hemolysis [44]. To overcome these limitations, we developed BV nano-liposomes (BV@NLs) by encapsulation using lecithin and chitosan and evaluate the biological activity *in vitro* against three cell lines.

The current results indicated that the synthesized BV@NLs have an average particle size of 230.9 ± 5.21 nm and a ζ-potential of 46.5 ± 1.25 mV. The particle charge is well known as the stability determining factor of nanoparticles and the ζ-potential of $> +30$ mV and < -30 mV is ideal for the physical stability of any suspension [45]. The observed liposomes displayed a spherical shape, in agreement with the findings of Thompson and Singh [46](. The spontaneous mixing of BV and chitosan in an acetic acid solution resulted in the formation of compact nanocomposites with an overall positive surface charge, and the surface charge density was reflected in the measured zeta potential values. A similar range of zeta potentials (>+30 mV) has been obtained for salicylic acid and gentamicin or fungal chitosan-loaded nanoparticles in previous reports [1]. The FTIR analysis of chitosan/BV/lecithin nanoparticles confirmed the chitosan moiety [1], lecithin [47,48] and BV [18,49].

In the *in vitro* study, the synthesized BV@NLs were tested for their anticancer activity against HepG-2, MCF-7, and HCT-16 cell lines. The results showed that BV@NLs exhibited strong anticancer properties against the three examined cell lines compared to the crude BV. Additionally, BV@NLs were more effective against the HCT-116 cell line compared to HepG-2 and MCF-7. The anticancer activity of BV was reported in a series of reports [50,51]. Wehbe et al. [20] reported that BV is a complex mixture containing several pharmacological bioactive compounds, including peptides such as melittin, mast cell degranulating (MCD) peptides, apamin, and adolapin. It also contains enzymes mainly hyaluronidase, phospholipase A2 (PLA2), and bioactive amines like dopamine, and histamine along with some amino acids, volatile compounds, and minerals. These components may be differing according to several factors such as strain, age, caste, season, and collection methods [39,52].

Melittin as the main compound is water soluble and has cationic, linear, amphipathic, and hemolytic properties consisting of 26 amino acids with 2840 Da, cationic, hemolytic, linear, and amphipathic properties [53]. This compound showed several biological activities and was recognized as an anti-parasitic antifungal, antibacterial, antiviral, and anti-tumor peptide owing to its activity as a non-selective cytolytic peptide which disturbs the physical and chemical structure of all eukaryotic and prokaryotic cell membranes [54]. In the current study, BV and BV@NLs showed anticancer effect against the examined cell lines since it inhibits carcinoma cell proliferation and suppresses tumor growth. It was reported that BV stimulates the local cellular immune responses in the lymph nodes [55,56].

The mechanisms of BV involve necrosis, apoptosis, and lysis of tumor cells [57,58]. Recently, BV was reported to induce human leukemic cell apoptosis via the induction of caspase-3 and Bcl-2 expression by the down-regulation of mitogen-activated signal pathways [43]. Additionally, BV also has an apoptotic effect through caspase-3 activation in synovial fibroblasts [59] and the inhibition of cyclooxygenase-2 expression in human lung cancer cells [56,60]. Our study showed that colorectal carcinoma cells (HCT-116) were the most affected by the BV and BV@NLs compared to the other two cell lines (HepG-2 and MCF-7). These results were similar to those reported by Duarte et al. [61] and suggested that BV has a selective cytotoxic effect on cancer cells [62]. Moreover, BV@NLs were more effective than crude BV. This action may be due to the effect of liposomes, which are non-ionic surface-active vesicles that can directly target cancer cells by increasing efficacy and reducing side effects [63].

Previous studies revealed that free melittin in BV can be toxic to normal cells but the formulation of melittin in nanoparticles appears unreactive against the vaginal reporter or epithelial cells *in vitro* [64]. Additionally, melittin nanoparticles were reported to interact with cells by traditional lipid-to-lipid membrane hemifusion events which appear similar to that occur between the bilayers of liposomes lipid membrane [65]. In general, the potential anticancer role of BV@NLs may be due to several reasons including (1) the improvement of its delivery and release [1,39], the maintenance of sustained release and efficacy for a long time [66], (2) the extent of its biological activity [67], (3) the increasing of immune response and cytokines [68], (4) increase the bioactivity against cancer cells [1]. Additionally, Strait [69] reported that loading BV on chitosan nanoparticles leads to the activation of anticancer activity against cervical cancer and induces signs of serious apoptosis in a dose- and time-dependent manner.

The results showed that BV@NLs elevated the expression of Bax mRNA and reduced the expression of Bcl-2 mRNA and P53 mRNA compared to the control cells. Bcl-2 and Bx are the two key regulators in the apoptosis process of mitochondria [70] and Bcl-2 protects the cell from oxidative damage and its overexpression can suppress lipid peroxidation completely [71]. The down-regulation of Bcl-2 and the up-regulation of Bax reported herein in the cancer cell lines exposed to BV or BV@NLs suggested that these agents killed cancer cell lines through the apoptosis mechanism mainly via these genes. Similar findings were reported by Jo et al. [72] who showed that BV inhibited the growth of human ovarian cancer cells and inhibits the expression of Bcl-2 while increasing the pro-apoptotic Bax. Additionally, BV was reported to decrease Bcl-2 expression and increase Bax, caspase-3,8,9 which regulate NF- κB in colon cancer [73]. Furthermore, Abdulmalek et al. [74] showed that BV-CSNPs increased Bax expression in liver cancer cells compared to crude BV. The increase in Bax expression along with the decrease in Bcl-2 expression suggested that BV@NLs blocked the epidermal growth factor receptor (EGFR) pathway leading to the

downstream of MEK/ERK pathway which is a signal transduction pathway critical for cell proliferation [75]. Previous reports showed that BV has multi-potential effects on signaling pathways such as the MAPK pathway which was accompanied by the increase of p38-MAPK phosphorylation [76]. Taken together, the reduction of Bcl-2 mRNA expression and elevation of Bax mRNA are vital in promoting apoptosis [77].

5. Conclusion

The current study showed that the anticancer activity of BV can be enhanced through the formation of the BV-nanoliposome (BV@NLs) complex. The prepared BV@NLs showed a spherical shape with particle size of 230.9 ± 5.21 nm and ζ-potential of 46.5 ± 1.25 mV. BV@NLs showed a potent anticancer against HepG-2, MCF-7, and HCT-116 cell lines. BV@NLs was more effective than crude BV and the concentration of 100 μg was very effective than the lower concentrations. BV@NLs showed a selective cytotoxic effect on cancer cells since they were more effective against HCT-116 cell lines. The mode of action of BV@NLs included the modulation of apoptosis-related gene (BAX, Bcl-2, and P53) expression. Therefore, the preparation of BV@NLs may be promising in the application of BV in cancer treatment.

CRediT authorship contribution statement

This work was carried out in collaboration between all authors. Authors Alaa Abd El-Gawad, TM El-Messery, ME Hassan and AA El-Nekeety carried out the experimental work and shared in writing the first draft of the manuscript. Author MAA Kenawy managed the literature searches, performed the statistical analysis and shared in writing the first draft of the manuscript. Author MA Abdel-Wahhab wrote the protocol, managed the project, managed the analyses of the study and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the National Research Centre, Dokki, Cairo, Egypt project # 13050302, and the Faculty of Science, Ain Shams University, Cairo, Egypt.

References

[1] A.I. Alalawy, H.A. El Rabey, F.M. Almutairi, A.A. Tayel, M.A. Al-Duais, N.S. Zidan, M. Sakran, Effectual anticancer potentiality of loaded bee venom onto fungal chitosan nanoparticles, Int. J. Polym. Sci. (2020), 2785304, https://doi.org/10.1155/2020/2785304.
[2] S.C. Pak, Health benefits and uses in medicine of bee venom, in: J.M. Alvarez-Suarez (Ed.), Bee Products - Chemical and Biological Properties, Springer Int. Pub. AG, Cham, Switzerland, 2017, pp. 287–306, 2017.
[3] H. Raghuraman, A. Chattopadhyay, Melittin: a membrane-active peptide with diverse functions, Biosci. Rep. 27 (4–5) (2017) 189–223.
[4] S.D.A. El-Wahab, L.H. Eita, The effectiveness of live bee sting acupuncture on depression, J. Nurs. Health Sci. 4 (2015) 19–27.
[5] P.M. Fratellone, Apitherapy products for medicinal use, J. Nutr. Food Sci. 2015 (2015) 5.
[6] A.G. Hegazi, Role of cytokines in bee venom therapy-Part I, Apither. Rev. Issue 3 (2009).
[7] A.G. Hegazi, Medical importance of bee products, ARI B L M/BEE Sci. 12 (4) (2012) 136–146.
[8] E. Jamasbi, S.S. Lucky, W. Li, M.A. Hossain, P. Gopalakrishnakone, F. Separovic, Effect of dimerized melittin on gastric cancer cells and antibacterial activity, Amino Acids 50 (8) (2018) 1101–1110, https://doi.org/10.1007/s00726-018-2587-6.
[9] C.C. Liu, H. Yang, L.L. Zhang, Q. Zhang, B. Chen, Y. Wang, Biotoxins for cancer therapy, Asian Pac. J. Cancer Prev. APJCP 15 (12) (2014) 4753–4758.
[10] A.E.B.M. El-Beltagy, H.I.H. Elsyyad, K.K. Abdelaziz, A.S. Madany, M.M. Elghazaly, Therapeutic role of *Annona muricata* fruit and bee venom against MNU-induced breast cancer in pregnant rats and its complications on the ovaries, Breast Cancer 13 (2021) 431–445, https://doi.org/10.2147/BCTT.S306971.
[11] N.Y. Kwon, S.H. Sung, H.K. Sung, J.K. Park, Anticancer activity of bee venom components against breast cancer, Toxins 1 (7) (2011) 460, https://doi.org/10.3390/toxins14070460. Jul 5.
[12] A. Aufschnaiter, V. Kohler, S. Khalifa, A. Abd El-Wahed, M. Du, H. El-Seedi, S. Büttner, Apitoxin and its components against cancer, neurodegeneration and rheumatoid arthritis: limitations and possibilities, Toxins 12 (2) (2020) 66, https://doi.org/10.3390/toxins12020066.
[13] S. Bae, H. Gu, M.G. Gwon, H.J. An, S.M. Han, S.J. Lee, J. Leem, K.K. Park, Therapeutic effect of bee venom and melittin on skin infection caused by *Streptococcus pyogenes*, Toxins 14 (10) (2022) 663, https://doi.org/10.3390/toxins14100663, 2022 Sep. 23.
[14] V. Herzig, Animal venoms-curse or cure? Biomedicines 9 (4) (2021) 413, https://doi.org/10.3390/biomedicines9040413.
[15] P. Shi, S. Xie, J. Yang, Y. Zhang, S. Han, S. Su, H. Yao, Pharmacological effects and mechanisms of bee venom and its main components: recent progress and perspective, Front. Pharmacol. 13 (2022), 1001553, https://doi.org/10.3389/fphar.2022.1001553.
[16] T.Y. Lin, C.L. Hsieh, Clinical applications of bee venom acupoint injection, Toxins 12 (10) (2020) 618, https://doi.org/10.3390/toxins12100618, 2020 Sep. 27.
[17] Y. Gülmez, A. Aydın, I. Can, S. Tekin, E. Cacan, Cellular toxicity and biological activities of honey bee (*Apis mellifera* L.) venom, Marmara Pharm. J. 21 (2017) 51–260.
[18] A.E. Saber, A.K. Abdelwahab, A.M. El Amir, M.I. Nassar, H.F. Zohdi, Bee venom loaded chitosan nanoparticles as treatment for amoebiasis in mice, J. Egypt. Soc. Parasitol. 47 (2) (2017) 443–458.
[19] B. Cheng, B. Thapa, K.C. Remant, P. Xu, Dual secured nano-melittin for the safe and effective eradication of cancer cells, J. Mater. Chem. B 3 (2015) 25–29.
[20] R. Wehbe, J. Frangieh, M. Rima, D. El Obeid, J.M. Sabatier, Z. Fajloun, Bee venom: overview of main compounds and bioactivities for therapeutic interests, Molecules 24 (16) (2019) 2997, https://doi.org/10.3390/molecules24162997.
[21] J. Ferlay, M. Ervik, F. Lam, M. Colombet, L. Mery, M. Piñeros, et al., Global Cancer Observatory: Cancer Today, International Agency for Research on Cancer, Lyon, 2021. February 2021), https://gco.iarc.fr/today.
[22] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2021: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 71 (3) (2021) 209–249, https://doi.org/10.3322/caac.21660.
[23] WHO, Global Health Estimates 2020: Deaths by Ccause, Age, Sex, by Country and by Region, 2000-2019, WHO, 2020 (who.int/data/gho/data/themes/mortality-andglobal-health-estimates/ghe-leadingcauses-of-death). (Accessed 11 December 2020).
[24] F. Bray, M. Laversanne, E. Weiderpass, I. Soerjomataram, The ever-increasing importance of cancer as a leading cause of premature death worldwide, Cancer 127 (16) (2021) 3029–3030, https://doi.org/10.1002/cncr.33587.
[25] C. de Martel, D. Georges, F. Bray, J. Ferlay, G.M. Clifford, Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis, Lancet Global Health 8 (2) (2020) e180–e190.
[26] A.C. Corrêa, P.R. Pereira, V.M. Paschoalin, Preparation and characterization of nanoliposomes for the entrapment of bioactive hydrophilic globular proteins, J. Vis. Exp. 150 (2019), e59900, https://doi.org/10.3791/59900.
[27] X. Wang, Y. Wang, Z.G. Chen, D.M. Shin, Advances of cancer therapy by nanotechnology, Cancer Res. Treat. 41 (1) (2009) 1–11.
[28] T.M. Taylor, P.M. Davidson, B.D. Bruce, J. Weiss, Liposomal nanocapsules in food science and agriculture, Crit. Rev. Food Sci. Nutr. 45 (2005) 587–605.
[29] S. Khorasany, M. Danaei, M.R. Mozafari, Nanoliposome technology for the food and nutraceutical industries, Trends Food Sci. Technol. 79 (2018) 106–115.
[30] A. Zarrabi, M. Alipoor Amro Abadi, S. Khorasani, M.R. Mohammadabadi, A. Jamshidi, S. Torkaman, E. Taghavi, M.R. Mozafari, B. Rasti, Nanoliposomes and tocosomes as multifunctional nanocarriers for the encapsulation of nutraceutical and dietary molecules, Molecules 25 (3) (2020) 638, https://doi.org/10.3390/molecules25030638. Feb 1.
[31] S. Ferreira dos, S.C. Lopes, M.S. Franco, M.C. Oliveira, pH-sensitive liposomes for drug delivery in cancer treatment, Ther. Deliv. 4 (9) (2013) 1099–1123.
[32] A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: classification, preparation, and applications, Nanoscale Res. Lett. 8 (2013) 102, https://doi.org/10.1186/1556-276X-8-102.
[33] F. Szoka, D. Papahadjopoulos, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, Proc. Natl. Acad. Sci. U. S. A 75 (9) (1987) 4194–4198.
[34] O. Zumbuehl, H.G. Weder, Liposomes of controllable size in the range of 40 to 180 nm by defined dialysis of lipid/detergent mixed micelles, Biochim. Biophys. Acta 640 (1) (1981) 252–262.
[35] M. Gültekin-Özgüven, A. Karadağ, S. Duman, Ş. Özkal, B. Özçelik, Fortification of dark chocolate with spray dried black mulberry (*Morus nigra*) waste extract

encapsulated in chitosan-coated liposomes and bioaccessability studies, Food Chem. 15 (2016) 205–212, 201.
[36] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J. Immunol. Methods 89 (2) (1986) 271–277, https://doi.org/10.1016/0022-1759(86)90368-6.
[37] M.A. Abdel-Wahhab, M.A. Hassan, A.A. El-Nekeety, S.H. Abdel-Aziem, N. S. Hassan, I. Jaswir, H.M. Salleh, Zinc citrate loaded whey protein nanoparticles ameliorates CCl_4-induced testicular injury via the regulation of Nrf2-Keap1 antioxidative signaling pathway, J. Drug Deliv. Sci. Technol. 61 (2021), 102322.
[38] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method, Methods 25 (4) (2001) 402–408.
[39] A. Khalil, B.H. Elesawy, T.M. Ali, O.M. Ahmed, Bee venom: from venom to drug, Molecules 26 (16) (2021) 4941, https://doi.org/10.3390/molecules26164941.
[40] M.B. Pucca, F.A. Cerni, I.S. Oliveira, T.P. Jenkins, L. Argemí, C.V. Sørensen, S. Ahmadi, J.E. Barbosa, A.H. Laustsen, Bee updated: current knowledge on bee venom and bee envenoming therapy, Front. Immunol. 10 (2019) 2090, https://doi.org/10.3389/fimmu.2019.02090.
[41] A. Kurek-Górecka, K. Komosinska-Vassev, A. Rzepecka-Stojko, P. Olczyk, Bee venom in wound healing, Molecules 26 (1) (2020) 148, https://doi.org/10.3390/molecules26010148.
[42] S. Sangboonruang, K. Kitidee, P. Chantawannakul, K. Tragoolpua, Y. Tragoolpua, Melittin from *Apis florae* venom as a promising therapeutic agent for skin cancer treatment, Antibiotics (Basel) 9 (8) (2020) 517, https://doi.org/10.3390/antibiotics9080517.
[43] J. Zhao, W. Hu, Z. Zhang, Z. Zhou, J. Duan, Z. Dong, H. Liu, C. Yan, Bee venom protects against pancreatic cancer via inducing cell cycle arrest and apoptosis with suppression of cell migration, J. Gastrointest. Oncol. 13 (2) (2022) 847–858.
[44] W. DeGrado, G. Musso, M. Lieber, E. Kaiser, F. Kézdy, Kinetics and mechanism of hemolysis induced by melittin and by a synthetic melittin analogue, Biophys. J. 37 (1982) 329–338.
[45] M.A. Mahmoud, M.A. El-Bana, S.M. Morsy, E.A. Badawy, A.E. Farrag, M. A. Badawy, A.M. Abdel-Wahhab, M.A. El-Dosoky, Synthesis and characterization of berberine-loaded chitosan nanoparticles for the protection of urethane-induced lung cancer, Int. J. Pharm. 61 (2022), 121652, https://doi.org/10.1016/j.ijpharm.2022.121652.
[46] A.K. Thompson, H. Singh, Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer, J. Dairy Sci. 89 (2) (2006) 410–419.
[47] F. Hosseini, M.S. Sadjadi, Synthesis and characterization of soy lecithin coated magnetic iron oxide nanoparticles for magnetic resonance imaging applications, Orient. J. Chem. 32 (6) (2016). http://www.orientjchem.org/?p=26407.
[48] W. Michał, D. Ewa, C. Tomasz, Lecithin-based wet chemical precipitation of hydroxyapatite nanoparticles, Colloid Polym. Sci. 293 (2015) 1561–1568.
[49] M.I. Hassan, S.I. El-dek, A.F. Mohamed, K.A. Abdelwahab, *In vitro* assessment of antimicrobial activity of chitosan nanoparticles loaded with the honeybee, *Apis mellifera* venom. Egypt, Acad. J. Biol. Sci. 12 (3) (2019) 85–103.
[50] N. Orsolić, Bee venom in cancer therapy, Cancer Metastasis Rev. 31 (2011) 173–194.
[51] F. Şengül, H. Vatansev, Overview of apitherapy products: anti-cancer effects of bee venom used in apitherapy, Int. J. Tradit. Complement. Med. Res. 2 (1) (2021) 36–48.
[52] A.A. Abd El-Wahed, S.A.M. Khalifa, B.Y. Sheikh, M.A. Farag, A. Saeed, F.A. Larik, U. Koca-Caliskan, M.F. AlAjmi, M. Hassan, H.A. Wahabi, M.E. F Hegazy, Bee venom composition: from chemistry to biological activity, in: Studies in Natural Products Chemistry vol. 60, Elsevier B.V., Amsterdam, The Netherlands, 2018, pp. 459–484.
[53] I. Rady, I.A. Siddiqui, M. Rady, H. Mukhtar, Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy, Cancer Lett. 402 (2017) 16–31.
[54] N. Do, G. Weindl, L. Grohmann, M. Salwiczek, B. Koksch, H.C. Korting, M. Schäfer-Korting, Cationic membrane-active peptides-anticancer and antifungal activity as well as penetration into human skin, Exp. Dermatol. 23 (2014) 326–331.
[55] X. Liu, D. Chen, L. Xie, R. Zhang, Effect of honey bee venom on proliferation of K1735M2 mouse melanoma cells *in-vitro* and growth of murine B16 melanomas *in-vivo*, J. Pharm. Pharmacol. 54 (2002) 1083–1089.
[56] P. Premratanachai, C. Chanchao, Review of the anticancer activities of bee products, Asian Pac. J. Trop. Biomed. 4 (5) (2014) 337–344.
[57] F.Z. Sharkawi, S.S. Saleh, A.F.M. Sayed, Potential anticancer activity of snake venom, bee venom and their components in liver and breast carcinoma, Int. J. Pharma Sci. Res. 6 (2015) 3224–3235.
[58] P. Shaw, N. Kumar, D. Hammerschmid, A. Privat-Maldonado, S. Dewilde, A. Bogaerts, Synergistic effects of melittin and plasma treatment: a promising approach for cancer therapy, Cancers 11 (2019) 1109.
[59] P. Soltan-Alinejad, H. Alipour, D. Meharabani, K. Azizi, Therapeutic potential of bee and scorpion venom phospholipase A2 (PLA2): a Narrative Review, Iran. J. Med. Sci. 47 (4) (2022) 300–313.
[60] C. Tipgomut, A. Wongprommoon, E. Takeo, T. Ittiudomrak, S. Puthong, C. Chanchao, Melittin induced G1 cell cycle arrest and apoptosis in chago-K1 human bronchogenic carcinoma cells and inhibited the differentiation of THP-1 cells into tumour-associated macrophages, Asian Pac. J. Cancer Prev. APJCP 19 (2018) 3427–3434.
[61] D. Duarte, S.I. Falcão, I. El Mehdi, M. Vilas-Boas, N. Vale, Honeybee venom synergistically enhances the cytotoxic effect of CNS drugs in HT-29 colon and MCF-7 breast cancer cell lines, Pharmaceutics 14 (3) (2022) 511, https://doi.org/10.3390/pharmaceutics14030511.
[62] K. Daniluk, M. Kutwin, M. Grodzik, M. Wierzbicki, B. Strojny, J. Szczepaniak, J. Bałaban, M. Sosnowska, A. Chwalibog, E. Sawosz, S. Jaworski, Use of selected carbon nanoparticles as melittin carriers for MCF-7 and MDA-MB-231 human breast cancer cells, Materials 13 (1) (2019) 90, https://doi.org/10.3390/ma13010090.
[63] L. Kanaani, I. Javadi, M. Ebrahimifar, H.E. Shahmabadi, A.A. Khiyavi, T. Mehrdiba, Effects of cisplatin-loaded niosomal nanoparticles on BT-20 human breast carcinoma cells, Asian Pac. J. Cancer Prev. APJCP 18 (2017) 365–368.
[64] J.L. Hood, A.P. Jallouk, N. Campbell, L. Ratner, S.A. Wickline, Cytolytic nanoparticles attenuate HIV-1 infectivity, Antivir. Ther. 18 (1) (2013) 95–103.
[65] R. Bartelds, M.H. Nematollahi, T. Pols, M.C.A. Stuart, A. Pardakhty, G. Asadikaram, B. Poolman, B. Niosomes, An alternative for liposomal delivery, PLoS One 13 (4) (2018), e0194179, https://doi.org/10.1371/journal.pone.0194179. Apr 12.
[66] J.H. Park, B.K. Yim, J.H. Lee, S. Lee, T.H. Kim, Risk associated with bee venom therapy: a systematic review and meta-analysis, PLoS One 10 (2015), e0126971.
[67] M. Qiao, D. Chen, T. Hao, X. Zhao, H. Hu, X. Ma, Effect of bee venom peptide-copolymer interactions on thermosensitive hydrogel delivery systems, Int. J. Pharm. 345 (2007) 116–124.
[68] J. Lee, Y.M. Kim, J.H. Kim, C.W. Cho, J.W. Jeon, J.K. Park, S.H. Lee, B.G. Jung, B. J. Lee, Nasal delivery of chitosan/alginate nanoparticle encapsulated bee (*Apis mellifera*) venom promotes antibody production and viral clearance during porcine reproductive and respiratory syndrome virus infection by modulating T cell related responses, Vet. Immunol. Immunopathol. 200 (2018) 40–51.
[69] J.E. Strait, Nanoparticles Loaded with Bee Venom Kill HIV, Washington News Room, Washington University, St. Louis, Washington, USA, 2013. Available online: https://source.wustl.edu/2013/03/nanoparticles-loaded-with-bee-venom-kill-hiv/. (Accessed 7 March 2013).
[70] L. Lalier, F. Vallette, S. Manon, Bcl-2 family members and the mitochondrial import machineries: the roads to death, Biomolecules 12 (2022) 162.
[71] J.C. Reed, Double identity for proteins of the Bcl-2 family, Nature 387 (1997) 773–776.
[72] M. Jo, M.H. Park, P.S. Kollipara, B.J. An, H.S. Song, S.B. Han, J.H. Kim, M.J. Song, J.T. Hong, Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway, Toxicol. Appl. Pharmacol. 258 (2012) 72–81.
[73] J. Zheng, H.L. Lee, Y.W. Ham, H.S. Song, J. Song, J.T. Hong, Anti-cancer effect of bee venom on colon cancer cell growth by activation of death receptors and inhibition of nuclear factor kappa B, Oncotarget 6 (2015) 44437–44451.
[74] S. Abdulmalek, N. Mostafa, M. Gomaa, M. El-Kersh, A.I. Elkady, M. Balbaa, Bee venom-loaded EGFR-targeting peptide-coupled chitosan nanoparticles for effective therapy of hepatocellular carcinoma by inhibiting EGFR-mediated MEK/ERK pathway, PLoS One 17 (8) (2022), e0272776, https://doi.org/10.1371/journal.pone.0272776.
[75] K. Roovers, R.K. Assoian, Integrating the MAP kinase signal into the G1 phase cell cycle machinery, Bioessays 22 (2000) 818–826.
[76] J.E. Huh, J.W. Kang, D. Nam, Y.H. Baek, D.Y. Choi, D.S. Park, J.D. Lee, Melittin suppresses VEGF-A-induced tumor growth by blocking VEGFR-2 and the COX-2-mediated MAPK signaling pathway, J. Nat. Prod. 75 (2012) 1922–1929.
[77] F.F. Lem, B.E. Cheong, P.L. Teoh, *Ruellia tuberosa* ethyl acetate leaf extract induces apoptosis and cell cycle arrest in human breast cancer cell line, MCF-7, Sci. Pharm. 90 (2022) 44, https://doi.org/10.3390/scipharm90030044.
[78] I. Wiame, S. Remy, R.S. Swennen, L. Agi, Irreversible heat inactivation of DNaseI without RNA degradation, Biotechniques 29 (2000) 252–256.
[79] H. He, J. Sun, X. Huang, Expression of caspase-3, Bax and Bcl-2 in hippocampus of rats with diabetes and subarachnoid hemorrhage, Exp. Ther. Med. 15 (1) (2018) 873–877.
[80] V.S. Zununi, A. Barzegari, S.Y. Rahbar, A. Goreyshi, Y. Omidi, *Leuconostoc mesenteroides*-derived anticancer pharmaceuticals hinder inflammation and cell survival in colon cancer cells by modulating NF-κB/AKT/PTEN/MAPK pathways, Biomed. Pharmacother. 94 (2017) 1094–1100.
[81] S.L. Diao, H.P. Xu, B. Zhang, B.X. Ma, X.L. Liu, Associations of MMP-2, BAX, and Bcl-2 mRNA and protein expressions with development of atrial fibrillation, Med. Sci. Mon. 22 (2016) 1497–1507.
[82] N.C. Pollock, J.R. Ramroop, H. Hampel, M.A. Troester, K. Conway, J.J. Hu, J. L. Freudenheim, O.I. Olopade, D. Huo, E. Ziv, S.L. Neuhausen, P. Stevens, J. P. McElroy, A.E. Toland, Differences in somatic TP53 mutation type in breast tumors by race and receptor status, Breast Cancer Res. Treat. 192 (3) (2022) 639–648.
[83] M.M. El-Zayat, M.M. Eraqi, H. Alrefai, A.Y. El-Khateeb, M.A. Ibrahim, H. M. Aljohani, M.M. Aljohani, M.M. Elshaer, The antimicrobial, antioxidant, and anticancer activity of greenly synthesized selenium and zinc composite nanoparticles using ephedra aphylla extract, Biomolecules 11 (3) (2021) 470, https://doi.org/10.3390/biom11030470.