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In vitro cytotoxicity assessment of biosynthesized *Apis mellifera* bee venom nanoparticles (BVNPs) against MCF-7 breast cancer cell lines

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Abstract
In this work, we reported the synthesis of honey bee (*Apis mellifera*) venom-derived nanoparticles via a hydrothermal method. This method not only ensures the preservation of the bee venom's bioactive components but also enhances their potential stability, thus broadening the scope for their applications in the biomedicinal field. The synthesis method started with the homogenization suspension of bee venom, followed by its hydrothermal process to synthesize bee venom nanoparticles (BVNPs). The successful synthesis of BVNPs was characterized using various characteristic techniques such as Ultraviolet–visible (UV–Vis) spectroscopy, Fourier Transforms Infrared (FTIR) Spectroscopy, Zeta Potential (ZP), Liquid Chromatography-Mass Spectrometry (LCMS), and Transmission Electron Microscopy (TEM). The synthesis of BVNPs through biosynthesis is shown by the visible violet-brown color development at 347 nm by UV–Vis spectroscopy. FTIR analysis revealed the presence of several functional groups in the BVNPs, including alcohols (–OH), phenols (C_6H_5–), carboxylic acids (–COOH), amines (–NH_2, –NH–), aldehydes (–CHO), ketones (–CO–), nitriles (–CN), amides (–CO–N–), imines (–CNH–), esters (–COO–), and polysaccharides. These functional groups, as confirmed by their specific stretching and bending vibrational modes, contribute to the diverse biological activities of BVNPs, including cytotoxicity against MCF-7 breast cancer cells. The ZP of the BVNPs indicated good colloidal stability at – 45 mV. LCMS analysis confirmed the presence of major bioactive molecules, including melittin & apamin and TEM analysis shows the BVNPs exhibited a quasi-spherical shape with good dispersion, the average size was approximately 25 nm, with some being smaller (quantum dots) and interplanar spacing of 0.236 nm indicated a highly ordered crystalline structure. Moreover, the anticancer efficacy of the BVNPs was ascertained through in vitro assays against MCF-7 breast cancer cells, showing a dose-dependent cytotoxic effect. The findings of this study underscore the viability of hydrothermal synthesis in producing biologically active and structurally stable BVNPs, with a significant potential for anticancer activities.

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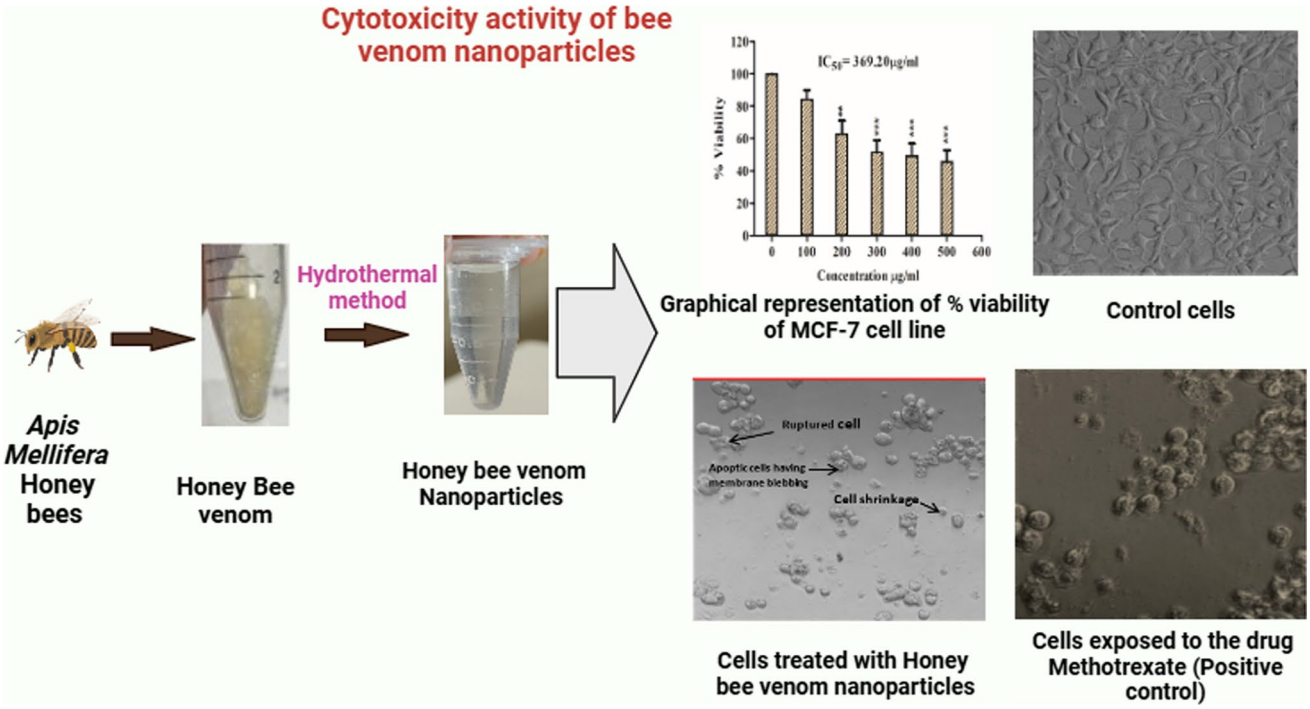
Graphical Abstract



Keywords *Apis mellifera* · Bee venom nanoparticles · Cytotoxicity · Hydrothermal synthesis · Anticancer activity · MCF-7 cells · Bio-medicinal applications

1 Introduction

Apis mellifera (Fig. 1) is the most common honeybee species utilized for agricultural pollination worldwide. All bee products, including venom and honey, have been used for thousands of years, and their medicinal powers or therapeutic purposes have been mentioned in historical texts [1, 2]. Apitherapy is a medicine that involves using honeybee products most importantly, bee venom (BV), also known as apitoxin. BV therapy; has been used as complementary and alternative therapy since ancient times [3]. But the mechanisms behind the venom's potential in battling cancer, particularly breast cancer, the most common cancer amongst women globally, remain largely veiled. Demystifying these molecular pathways and understanding how BV specifically targets cancer cells is crucial [4]. It holds the key to unlocking novel, effective cancer treatments derived from a natural source a resource readily available and affordable in many communities worldwide. BV, a complex mixture of various natural products derived from the honey bee, possesses a range of potential medicinal properties [5]. These properties can be attributed to various components within the venom, including peptides, enzymes, active amines, and non-peptide elements [5, 6]. Research suggests that BV therapy exhibits anti-inflammatory [7], apoptotic [8], anti-fibrotic [9], and anti-atherosclerotic effects [10]. It has been explored as a potential treatment for various diseases, including arthritis [11], amyotrophic lateral sclerosis [12], Parkinson's disease [13], Alzheimer's disease [14], liver fibrosis [15], and atherosclerosis [16]. Honey BV's major bioactive molecule is melittin, constituting half its dry weight. This 26-amino-acid peptide, positively charged and amphipathic, interacts with the cell membrane's phospholipids, forming tiny pores (~4.4 nm) that allow entry of other cytotoxic molecules. Both BV and melittin have shown promise against various cancers melanoma, lung, glioblastoma, leukemia, ovarian, cervical, and pancreatic demonstrating a preference for killing cancer cells over healthy ones [17]. This exciting research examines the way for further investigation of BV's potential as a natural weapon against cancer, armed with a deeper understanding of its molecular mechanisms [18].

Fig. 1 *Apis Mellifera* honey bees

Nanotechnology, the science of manipulating matter at the incredibly small scale of individual atoms and molecules, offers a promising avenue for cancer treatment. Nanoparticles (NPs), due to their unique physicochemical properties, hold immense potential as highly efficient delivery vehicles for therapeutic agents [19]. Their exceptional surface-to-mass ratio allows them to interact more effectively with their surrounding environment, making them ideal carriers for drugs. Physicochemical, thermal decomposition, electrochemical, microwave-aided, and various other techniques have been used to synthesize several NPs [20]. These methods are frequently expensive or produce by-products that endanger human health and the environment. Importantly, these tiny capsules can act as shielded containers, protecting their precious cargo from premature degradation in the bloodstream while releasing it upon reaching the targeted tumor site. Through careful design and optimization of size and surface characteristics, scientists can tailor NPs to navigate the body's complex network of blood vessels and specifically accumulate within the tumor microenvironment [21]. This targeted approach minimizes potential side effects on healthy tissues, maximizing the therapeutic impact. However, a formidable challenge arises in the form of drug resistance, where cancer cells develop mechanisms to evade the effects of chemotherapeutic agents [22]. This hurdle impedes the efficacy of both conventional and targeted therapies. Researchers are now actively investigating the development of multifunctional and multiplex NPs, essentially "smart bombs" loaded with multiple therapeutic agents and equipped with sophisticated targeting mechanisms [23]. These sophisticated nanocarriers hold the potential to overcome drug resistance, paving the way for more personalized and effective cancer treatment strategies [24]. An active field of academic and, more significantly, applied nanotechnology research is the synthesis of BVNPs. There was no literature on the physical or chemical processes used to synthesize BVNPs. Therefore, it is imperative to synthesize ecologically friendly methods for the production of NPs [25]. Exploiting the variety of natural or semi-synthetic drugs is a viable strategy to accomplish this goal. A compelling driving force behind the nascent discipline of "nanomedicine" is its capacity to significantly enhance therapeutic results in drug-related therapies [26]. One substance that is commonly brought up in nanomedical research is BVNPs, and conjugating BVNPs to certain drugs is one of the possibilities that is usually suggested. Many NPs may be produced chemically or physically, but green NPs synthesis has become more popular since it is less hazardous to the environment. The quality of produced NPs for use in future applications is determined by several factors [27]. The processes used, the amount of plant extract used, the kind of reduction agents used, the pH of the reaction mixture, the length of time, the temperature, the concentration, and the amount of light all affect how big or small the metal or metal oxide NPs that are synthesized during plant synthesis [28]. This research investigates unfamiliar ground, focusing on BVNPs synthesized via the hydrothermal method. For the first time, we evaluate their potential in the biomedicine field by assessing their anticancer activity. This study could unlock previously neglected avenues for developing natural, bee-derived therapeutic agents [14–16].

2 Materials and methods

2.1 Materials

Ethanol (Analytical grade (AR), purity of 98%), and deionized water of Grade I (extra pure) were procured from Charco Chemicals. Cell culture media and supplements included Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and a Penicillin–Streptomycin antibiotic mixture. The cell viability assays employed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and Dimethyl sulfoxide (DMSO) was used as a solvent in the experimental studies.

2.2 Acquisition and isolation of *Apis* mellifera venom

This study sourced *Apis mellifera* honey bees from a bee farm located in Pimpalgaon (B), Nashik, Maharashtra (India). Bee venom was manually extracted using an electric venom collector (EVC) model DPS-BVC-01. The EVC measured 310 × 220 × 50 mm and utilized a 9 V battery for operation. Following venom extraction, samples were subjected to freeze-drying and stored at 4–5 °C until further analysis.

2.3 Synthesis of BVNPs

One-pot hydrothermal synthesis as shown in Fig. 2 is a facile and eco-friendly method that can produce NPs with different shapes, sizes, and compositions by using water as a solvent and a single reactor under high temperature and pressure [20].

The principal aim of this study is to synthesize BVNPs from *Apis mellifera* BV using a hydrothermal method. Bee venom was collected during daylight hours, dried in the open air, and stored in dark glass bottles at 4–5 °C. The crude venom was purified using standard chemical methods and then dissolved in a solvent mixture of ethanol and deionized water [15, 16]. This suspension was subjected to ultrasonic treatment to form a homogeneous solution, followed by hydrothermal treatment at 120 °C for 4 h and continuous stirring with a magnetic stirrer for 2 h [29]. The resulting BVNPs were freeze-dried, characterized using UV–Vis, FTIR, ZP, LCMS and TEM, and found to be well-dispersed, spherical NPs with an average particle size of 26 ± 2 nm. These BVNPs have potential applications in anti-cancer therapy.

2.4 Cell lines

Human breast cancer cell line Michigan Cancer Foundation-7 (MCF-7) was procured from the National Centre for Cell Science, Pune, Maharashtra (India) and maintained in a T25 flask with Dulbecco's Modified Eagle Medium containing 10% FBS and 1% of Penicillin–Streptomycin antibiotic mix. Cell lines were incubated at 37 °C in the presence of 5% CO_2 in a humidified atmosphere [18].

2.5 MTT assay

Cytotoxicity studies were performed on the Human breast cancer MCF-7 cell line by using the MTT assay method [30], wherein the reduction of MTT by dehydrogenase enzyme in living cells liberates purple-colored formazan crystals which

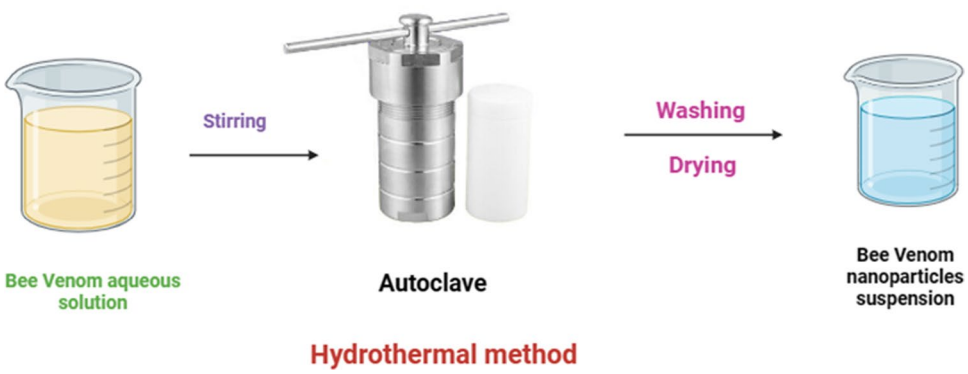


Fig. 2 Hydrothermal method for the synthesis of BVNPs

are directly proportional to the number of live cells. In brief, MCF-7 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well, grown in 100 µL of complete growth medium (90% DMEM with 10% FBS), and then incubated for 24 h. at 37 °C in 5% CO_2 environment. After incubation, the cells were exposed to different concentrations of BVNPs to be tested after removing the spent medium and further incubated for 24 h. Post incubation, MTT was added to the wells in a final concentration of 0.5 mg/mL and kept for at least 2 h. [30]. The formazon crystals so produced were dissolved by adding 100 µL of DMSO in each well and the absorbance was read at 570 nm. Three independent experiments were performed. Percent viability was calculated by the following formula;

$$\text{Percent cell viability} = \left(A_{570\,\text{sample}}/A_{570\,\text{Control}}\right) \times 100\,\%$$

3 Results and discussion

3.1 UV–Vis spectroscopy analysis

BVNPs showed a shift in the maximum absorbance to 347 nm as shown in Fig. 3 due to the presence of a large number of bioactive molecules. The redshift in the absorbance peak suggests changes in the electronic environment of the molecules due to the NPs formation [31]. This shift could be indicative of the successful encapsulation of BV molecules within the NPs, altering their optical properties.

The maximum absorbance (absorptive shift) of 327 nm (synthesized BVNPs) suggests that the electronic transitions taking place in the material have changed. Nevertheless, quantum confinement effects cause the electrical structure of NPs to alter as BV alters them. The distinct energy levels of NPs cause the absorption peak to redshift or increase in wavelength. This change implies that, in comparison to the bulk BV, the produced NPs have different electrical characteristics. The UV–Vis spectra offer important insights into the material's optical characteristics. This investigation is noteworthy as it implies that the NPs can be efficiently employed in uses that need UV absorption. The possible uses of honey BVNPs in drug administration, imaging, and other biological domains might be investigated further [32]. It is significant to notice that the absorption peak at 347 nm corresponds to the unique properties of the NPs, which might be related to their composition, size, or form. Comprehending these variables may improve future NP-based system design and optimization [33].

3.2 Fourier transform infrared (FTIR) analysis

The BVNPs have been studied using FTIR spectroscopy as shown in Fig. 4. The spectral profiling confirmed the functional groups of the BVNPs and displayed a distinct pattern of absorption bands, indicative of their primary bioactive components. The spectrum of a sample is a unique signature of its molecular composition and structure. The position of these

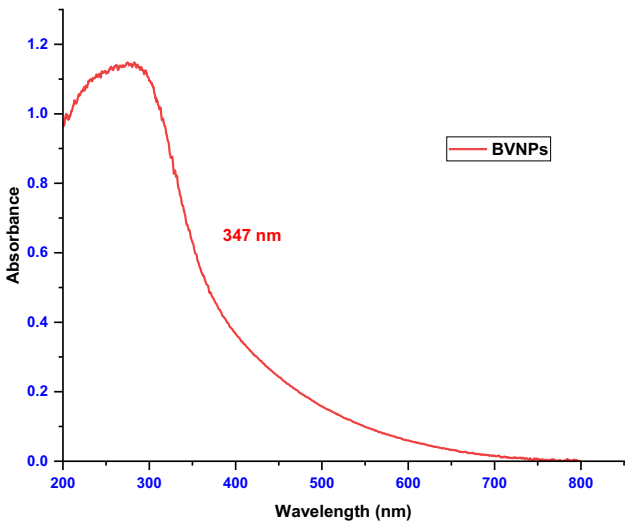


Fig. 3 UV–Vis spectra of BVNPs

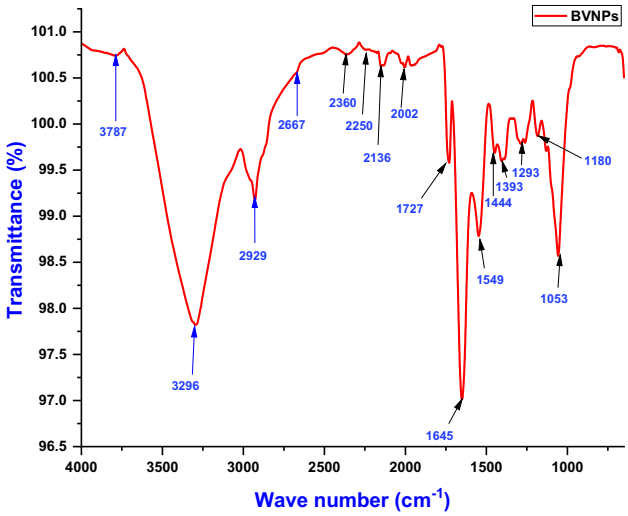


Fig. 4 FTIR spectra of the synthesized BVNPs

peaks facilitates the identification of bond types in the sample, which could correspond to various chemical bonds or functional groups within the BVNPs. Honey bees provide more than just delectable honey; their venom contains a complex mixture of bioactive molecules/compounds with a range of therapeutic uses. Deciphering the composition of this venom is vital for understanding its biological impacts and examining its potential [33, 34]. It serves as a potent tool for identifying the functional groups in BV, providing valuable insights into its bioactive chemical composition [35].

However, interpreting individual FTIR peaks (cm^{-1}) in isolation can be misleading. To truly investigate the inside of BVNPs: 3787 cm^{-1}; This peak confirms the presence of alcohol (–OH), phenol (C_6H_5–), or carboxylic acid (–COOH) groups, suggesting potential contributors to pain and inflammation [35]. 3296 cm^{-1}; This peaks of proteins or amines ($–NH_2$, –NH–, possibly enzymes involved in allergic reactions or tissue degradation [36]. 2929 & 2853 cm^{-1}; These peaks point towards aliphatic chains, likely representing fats & lipids that contribute to the venom's viscosity and potential immunomodulatory effects [35]. 2667 cm^{-1}; A possible indication of aldehydes (-CHO) or ketones (–CO–), which might play a role in the venom's antimicrobial activity [37]. 2360 & 2136 cm^{-1}; These peaks suggest the presence of nitriles (-CN) or amides (–CO–N–), hinting at potential neuroactive compounds affecting pain perception [38]. 2002 cm^{-1}; This could be linked to nitriles (–CN) or imines (–CNH–), potentially contributing to the venom's cytotoxic or antitumor activities [38]. 1727 cm^{-1}; Indicating esters (–COO–) or carboxylic acids (–COOH), possibly involved in inflammatory responses or enzymatic activities [35]. 1645 cm^{-1}; The characteristic peak of amides (–CO–NH–), further solidifying the presence of proteins, role in the venom's immunomodulatory & allergic reactions [39]. 1549 & 1444 cm^{-1}; These peaks suggest aromatic amines (-NH-) or imines (–CNH–), which might contribute to the venom's diverse biological effects [39]. 1393 cm^{-1}; This could be from aromatic compounds, potentially contributing to the venom's antioxidant or antibacterial properties [40]. 1293 cm^{-1}; Amines (–NH–, –N–, & NH_2), further suggesting a complex interplay of several bioactive molecules. 1053 & 1180 cm^{-1}; These peaks might indicate polysaccharides, possibly involved in the venom's immune system interactions [39, 40].

3.3 Zeta potential (ZP)

One of the key parameters in NPs characterization is the ZP, which in this case is – 45 mV (Fig. 5). ZP signifies the strength of the electrostatic repulsion or attraction between NPs in a suspension. It is a crucial factor influencing the stability of the suspension. A high ZP value (positive or negative) typically indicates a stable suspension, where the particles repel each other and resist clumping together. In general, NPs with ZP values exceeding + 30 mV or less than – 30 mV are considered highly stable. This is because the strong repulsive forces between the NPs prevent them from clumping together [41].

This stability is important for the delivery of the BV bioactive molecules or compounds, ensuring they remain effective until they reach their target. It is the key factor in the effectiveness of BVNPs as a delivery system for therapeutic compounds. A value of – 45 mV indicates a stable system, which is crucial for maintaining the effectiveness of the bioactive molecules in the BVNPs. This, combined with the potential for targeted delivery, makes BVNPs a promising area of research for new therapies. Our understanding of their mechanisms of action and how to best use them in various applications is still evolving, and further research is needed [41, 42].

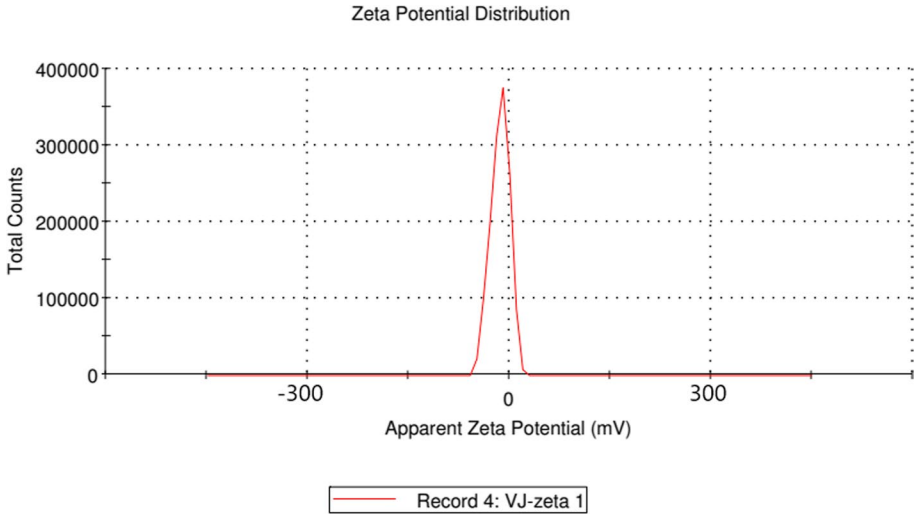


Fig. 5 ZP spectra of the synthesized BVNPs

3.4 Liquid chromatography mass spectrometry (LCMS) analysis

LCMS is a method or technique that combines liquid chromatography & mass spectrometry to separate, identify, and quantify the bioactive molecules or components of a complex mixture [43]. LCMS analysis of BVNPs can reveal the molecular weight, structure, and composition of the NPs and their BV constituents (Fig. 6). This can help to understand the mechanism of action and the pharmacokinetics of BVNPs in the body.

According to the study, the LCMS analysis of the BVNPs concluded the presence of several bioactive molecules or compounds. In the extract of the NPs, a total of 23 compounds including melittin and apamin were identified as mentioned in the supporting document. We discussed major chemical compounds present in the BVNPs such as

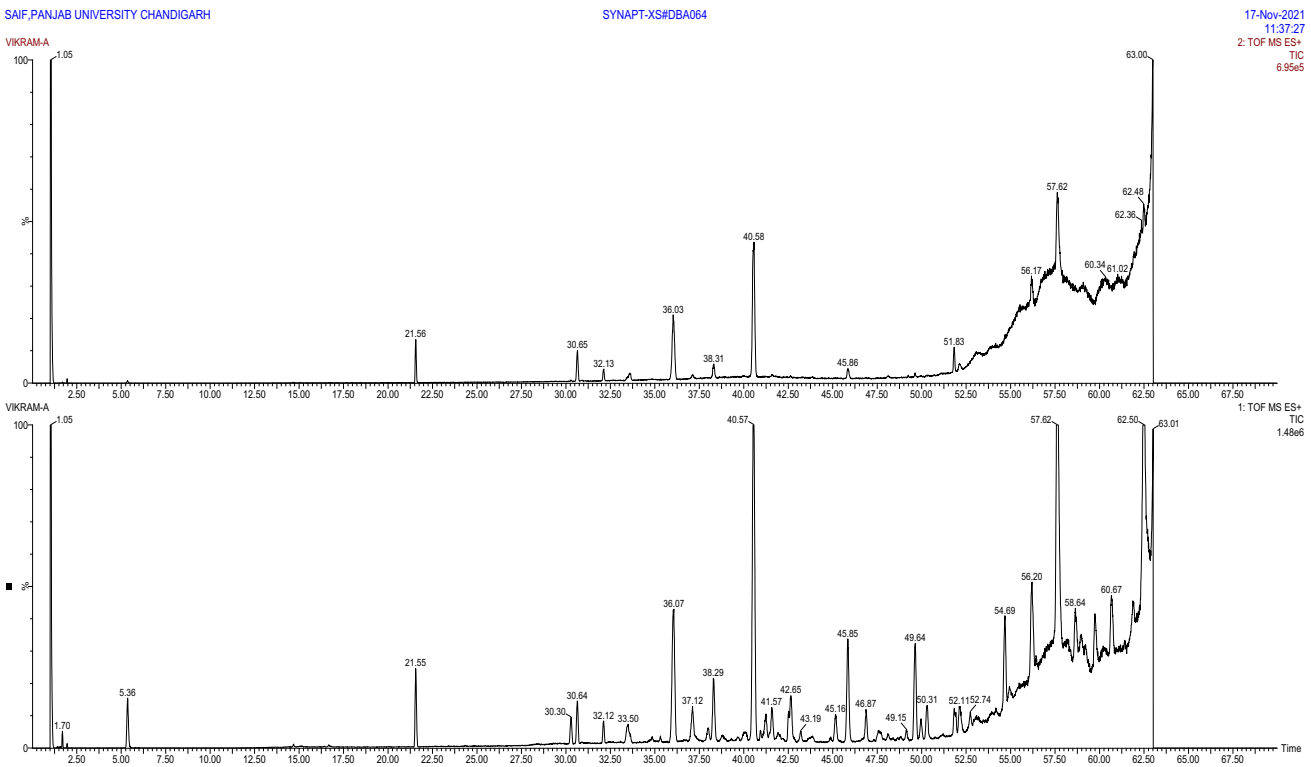


Fig. 6 LCMS spectra of the synthesized BVNPs

Table 1 BVNPs bioactive major molecule: Melittin properties

Polar peptide	Molar mass (Da)	Sequence	Formula	Observed charge states	The most abundant isotope mass (Da)
Melittin	2846.52	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2	$C_{131}H_{229}N_{39}O_{31}$	6+	475.3411
				5+	570.2043
				4+	712.5071
				3+	949.6701

The analysis identified $[M+5H]^{5+}$ at m/z 570.2043 as the most abundant ion, making it the chosen precursor ion for further analysis using product ion scan

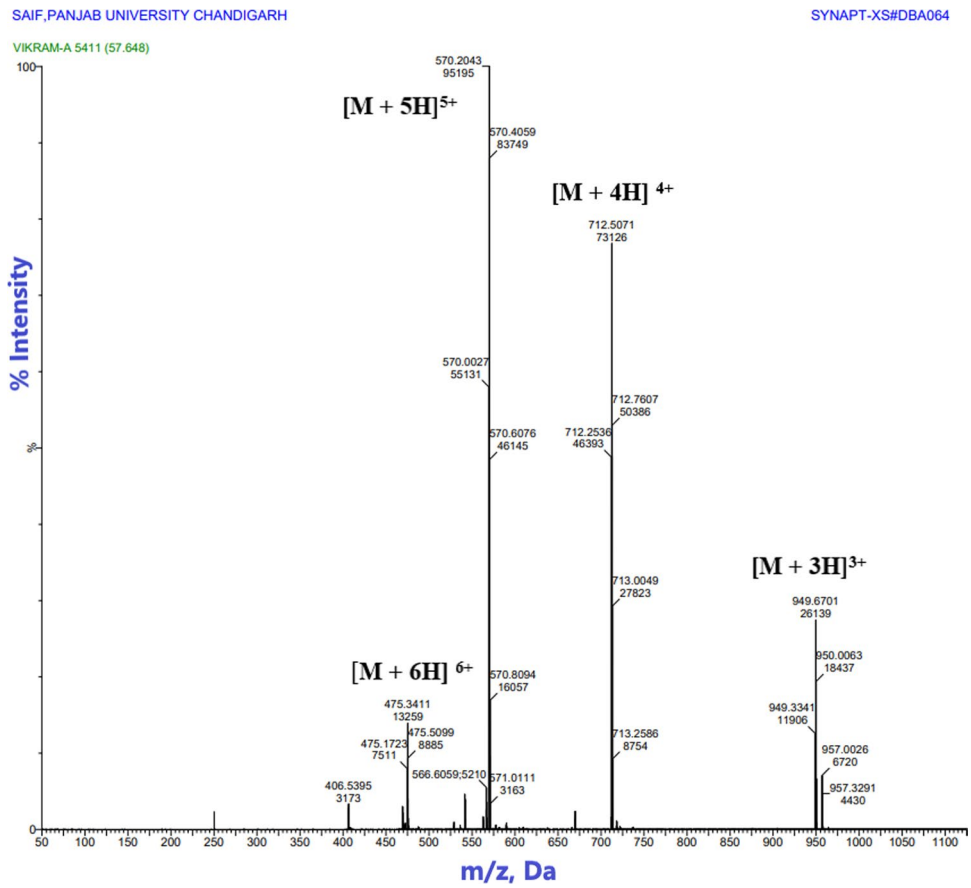


Fig. 7 High-resolution full scan mass spectrum of melittin

melittin [43] and apamin [44] from the LCMS data, here below is the complete description of these two bioactive chemical compounds.

3.4.1 Identifying melittin in BVNPs using MS:

Melittin is a polar peptide, that can be positively charged (protonated) at low pH and detected using MS in positive ion mode. When analyzing a small NPs sample extract with high-resolution MS (HRMS) in full scan mode with a narrow mass window (around ± 10 mDa), specific ions might indicate the presence of melittin [43–45]. These potential marker ions include $[M+6H]^{6+}$ at m/z 475.3411, $[M+5H]^{5+}$ at m/z 570.2043, $[M+4H]^{4+}$ at m/z 712.5071, and $[M+3H]^{3+}$ at m/z 949.6701 as shown in Table 1 (Fig. 7). The presence of these HR charged ions can be a strong indicator of melittin within the BVNPs.

Additionally, the second most abundant ion, $[M+4H]^{4+}$ at m/z 712.5071, served as the confirmation ion for melittin presence during multiple reactions monitoring quantitative analysis [46].

3.4.2 Identifying apamin in BVNPs using MS

Similar to melittin, apamin is another polar peptide, that becomes positively charged (protonated) at low pH and can be detected using MS in positive ion mode. When analyzing a small sample extract with HRMS in full scan mode with a narrow mass window (around ± 10 mDa), specific ions indicate the presence of apamin [44–46]. These potential marker ions include $[M+5H]^{5+}$ at m/z 406.0000, $[M+4H]^{4+}$ at m/z 507.7623, and $[M+3H]^{3+}$ at m/z 676.6818 as shown in Table 2 (Fig. 8). The detection of these HR charged ions strongly suggests the presence of apamin within the BVNPs.

The most abundant ion was $[M+4H]^{4+}$ at m/z 507.7623. Hence it was chosen as the precursor ion for the product ion scan.

Table 2 BVNPs bioactive molecule: Apamin properties

Polar peptide	Molar mass (Da)	Sequence	Formula	Observed charge states	The most abundant isotope mass (Da)
Apamin	2027.4	CNCKAPETALCARRCQQH	$C_{79}H_{131}N_{31}O_{24}S_4$	5 + 4 + 3 +	406.0000 507.7623 676.6818

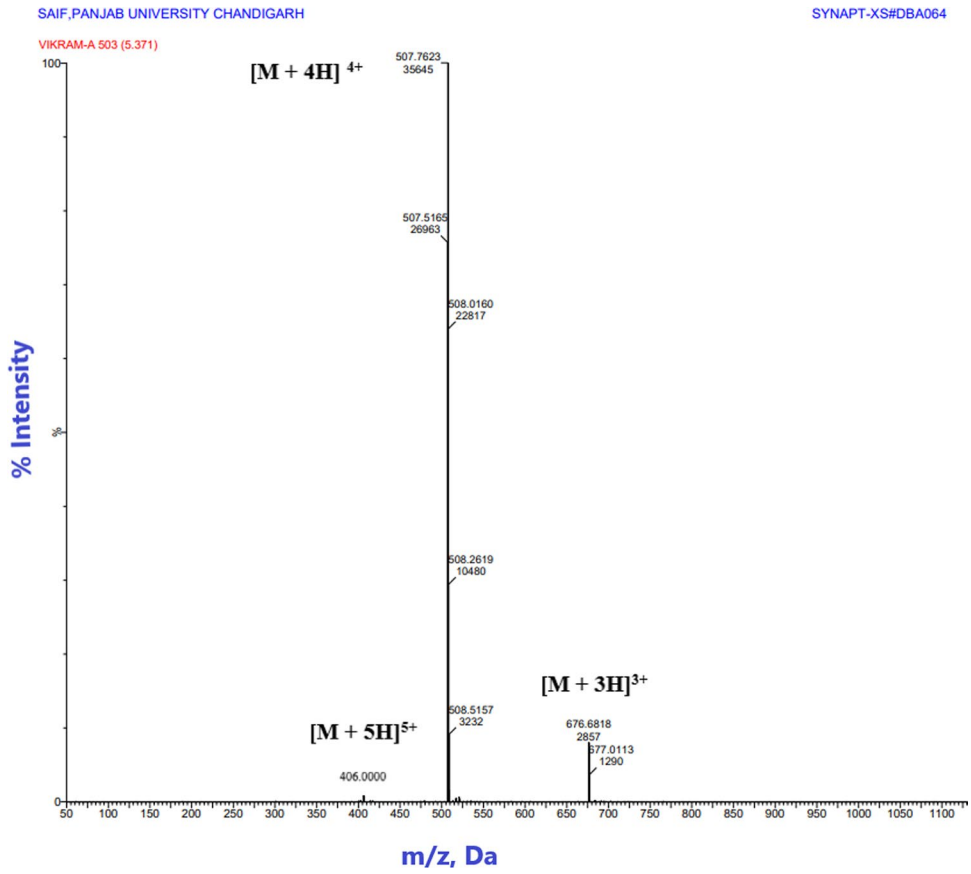


Fig. 8 High-resolution full scan mass spectrum of apamin

In multiple reactions monitoring quantitative analysis, the second most abundant ion at m/z 676.6818 ($[M+3H]^{3+}$) was chosen as the confirmation ion of apamin [44].

3.4.3 Transmission Electron Microscopy (TEM) analysis

It is a technique that uses a beam of electrons to produce an image of a sample at a very high magnification. TEM can reveal the morphology of the synthesized NPs [31, 32]. According to this study, BVNPs have a good dispersion, meaning they are evenly distributed in a solution. They also have a quasi-spherical type of morphology, meaning they are roughly spherical as shown in the Fig. 9.

The average size of BVNPs is around 25 nm, which is very small compared to the diameter of a human hair. Some of the NPs are even smaller, < 10 nm in size, and are called quantum dots. The images of BVNPs with an interplanar spacing of 0.236 nm suggest a highly ordered crystalline structure (Fig. 9). This spacing is indicative of the specific crystalline lattice and can be compared to known values for various nanomaterials to identify the composition of the NPs.

4 Cytotoxicity of BVNPs on human breast *cancer* cells

The antiproliferative effect of BVNPs against human breast adenocarcinoma (MCF-7) cells was evaluated using the MTT assay. The percent viability of MCF-7 cells was determined at various concentrations of BVNPs (0–500 µg/mL). The IC50 value, representing the concentration of BVNPs that inhibits cell growth by 50%, was calculated using a nonlinear regression analysis in GraphPad Prism. Each experiment was performed in triplicate (n = 3) to ensure the reliability and reproducibility of the results. The IC50 value obtained for BVNPs was 369.20 µg/mL, which was comparable to that of the standard drug methotrexate (IC50 = 56 µg/mL) (Fig. 10a). This indicates that BVNPs exhibit a significant antiproliferative effect against MCF-7 cells. The morphological changes observed in MCF-7 cells treated with BVNPs (Fig. 10b) further support their anti-cancer activity. Cells were treated with various concentrations of

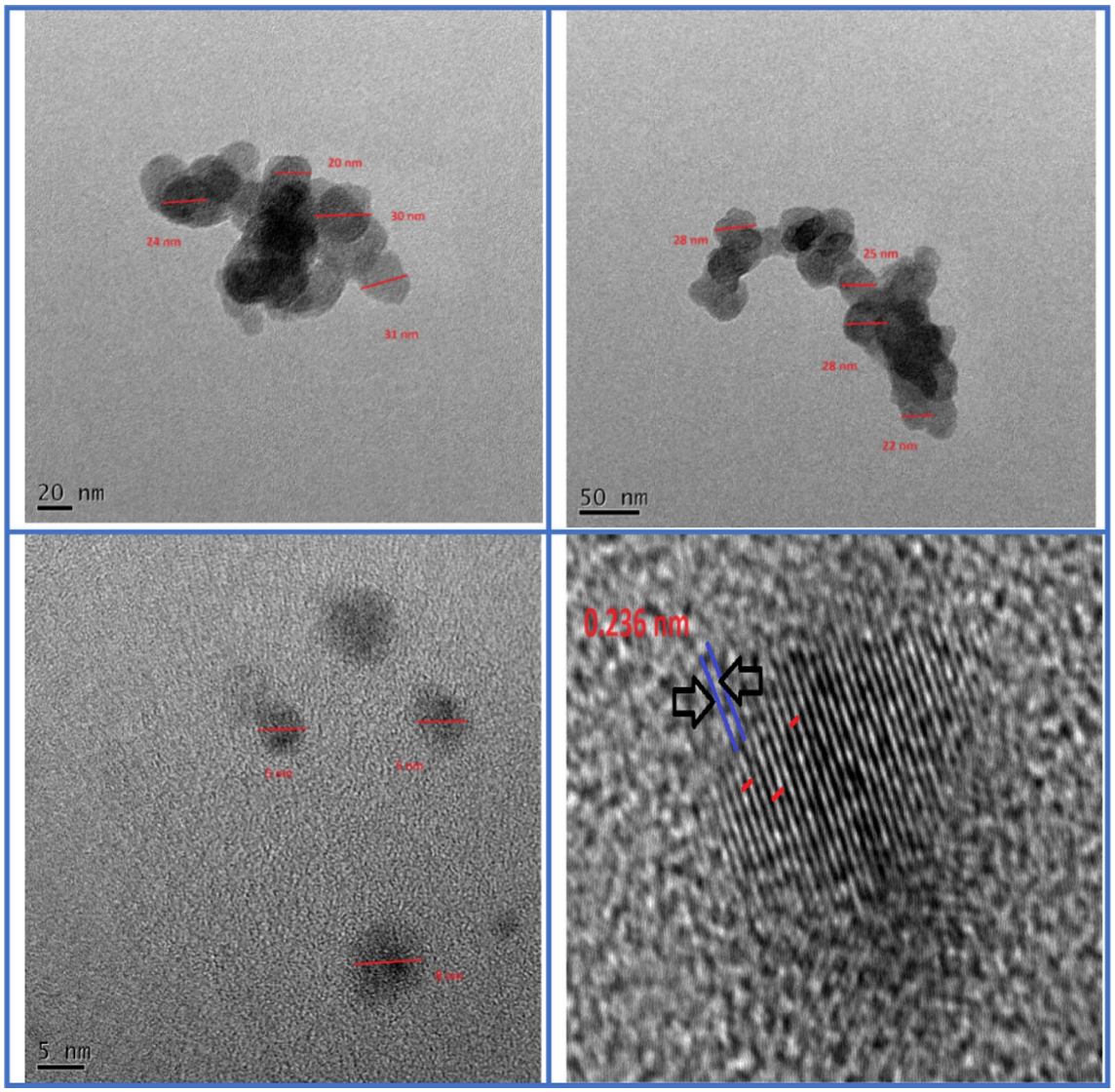


Fig. 9 TEM images of BVNPs

BVNPs (0–500 µg/mL) for 24 h. The results showed a significant dose-dependent decrease in cell viability, indicating the antiproliferative activity of BVNPs. Morphological changes in MCF-7 cells were observed under a microscope (20 × magnification) following treatment with BVNPs. The cells exhibited characteristic apoptotic features, including cell shrinkage, membrane blebbing, and nuclear fragmentation. These findings further support the anti-cancer activity of BVNPs. The morphological changes in MCF-7 cells due to BVNPs suggest that the cells might undergo apoptosis which has several morphological hallmarks like cell circularization, and shrinkage (Fig. 10c). The following figures reveal some of these hallmarks like cell rounding up and shrinkage. Some small circular bodies can be visualized indicating membrane blebbing. The same results can be seen in the case of methotrexate-treated cells (Fig. 10d) [47]. These BVNPs prove their potential to be antiproliferative. Honey BV and its components have been previously documented for their antimicrobial properties against various bacteria [48]. More recent research investigated the potential of honey BV and its bioactive molecules, including melittin, as anticancer agents [48, 49]. The study found that honey BV and melittin could suppress the growth of aggressive breast cancer subtypes by impacting the phosphorylation of EGFR and HER2 receptors. This study is the first to demonstrate the antiproliferative activity of BVNPs.

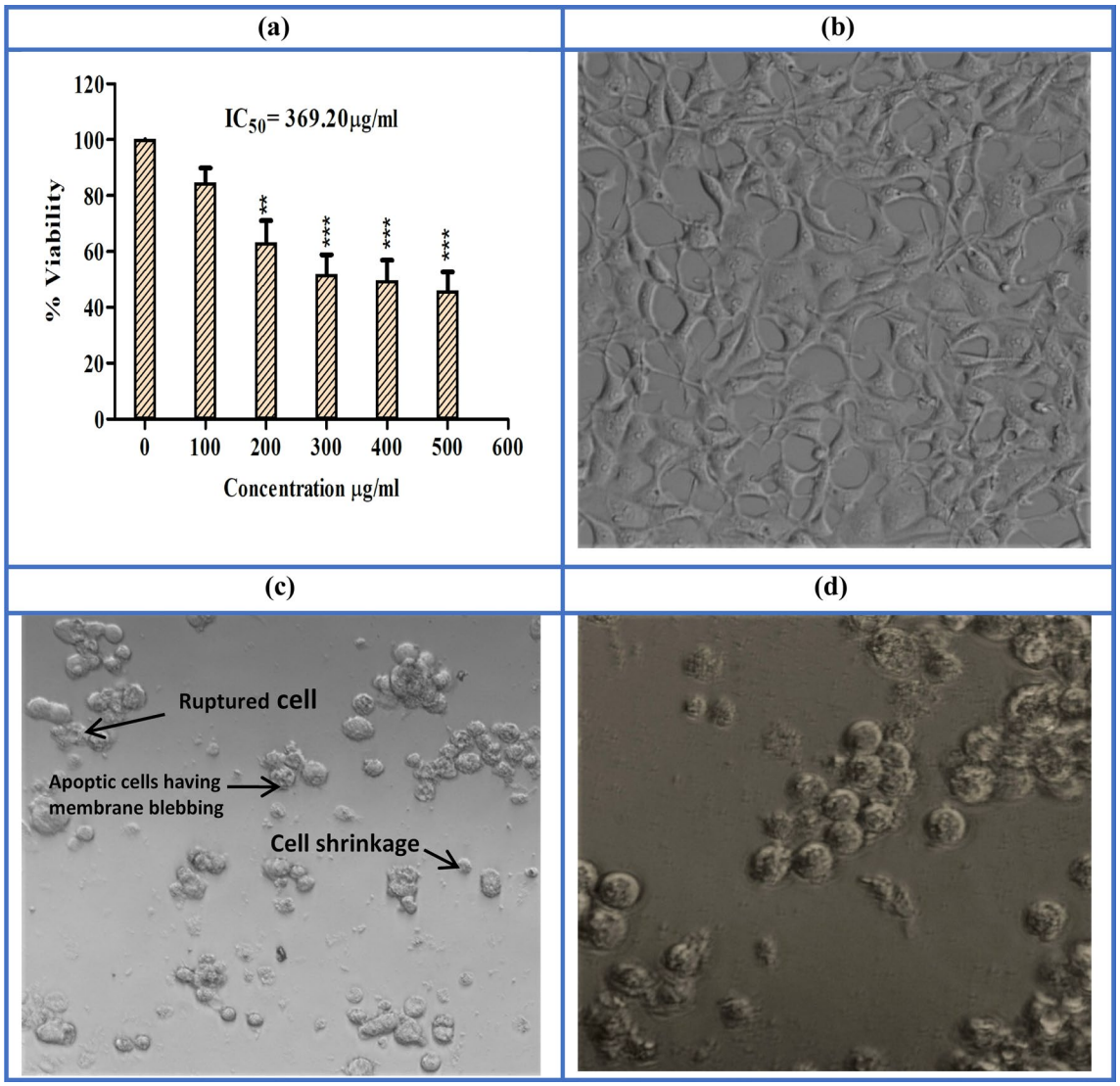


Fig. 10 **a** Graphical representation of % viability of MCF-7 cell line. Images of **b** Control cells, **c** Cells treated with BVNPs, showing morphological changes, and **d** cells exposed to the drug methotrexate (positive control)

5 Conclusion

In conclusion, this study successfully synthesized BVNPs using a facile hydrothermal method. The synthesized BVNPs exhibited a quasi-spherical morphology and were found to be monodispersed. Our findings revealed that the specific surface area of BVNPs significantly influenced their cytotoxic performance, with NPs possessing a higher specific surface area demonstrating superior efficacy. Notably, all types of BVNPs exhibited biocompatibility towards human breast cancer cell lines (MCF-7) at low concentrations (below 100 µg/mL). This suggests that nanomaterials of varying sizes and morphologies hold potential applications in biomedical fields, particularly in cancer diagnostics and therapy. Future research will focus on enhancing the biocompatibility of BVNPs through surface modification, aiming to further optimize their therapeutic potential. The promising results obtained in this study highlight the potential of BVNPs as a novel approach for the development of improved cancer diagnostics and therapeutics. This research represents a significant advancement in the application of nanotechnology for cancer research.

Author contributions D. L.: Conceptualization, data curation, writing—original draft. V. J.: writing—original draft. J. A.: Investigation, Formal analysis, writing—review & editing. A. B.: Supervision, resources. A. P.: Validation, editing. K. K.: conceptualization, validation, editing. A. D.: Methodology, investigation. A. K.: Resources, investigation.

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Data availability Yes, the research article will be available publically.

Declarations

Ethics approval and consent to participate No ethics approval was required for this study as it involved no harm to live vertebrates. The use of the human breast cancer cell line MCF-7 was conducted following institutional guidelines.

Competing interests The authors declare no competing interests.

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