

Calotropis procera-Derived Exosome-Like Nanoparticles as a Natural Delivery Platform for Polybia-MP1 in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death throughout the world and therefore, the development of safe and effective targeted therapeutic strategies is needed. The present study aimed to explore the feasibility of using Plant Derived Exosome like Nanoparticles (PDELNs) extracted from *Calotropis procera* as a natural nanocarrier for the delivery of the anti-cancer peptide Polybia-MP1. PDELNs were isolated based on a polyethylene glycol (PEG) method and characterized by SDS-PAGE, Western blotting, scanning electron microscopy, particle size analysis, polydispersity index, and zeta potential analysis to confirm the nanoscale physicochemical properties of the PDELNs. The sonication method was effective to encapsulate Polybia-MP1 with encapsulation efficiency of $42.1 \pm 2.13\%$ and sustained peptide release for 24 h. MP1-loaded PDELNs generated much higher cytotoxicity than free MP1 in HepG2 cells, decreasing the IC_{50} from 42.60 to 21.31 $\mu\text{g/mL}$. The therapeutic efficacy was further investigated in DEN/ CCl_4 -induced Wistar rat model of HCC, where administration of MP1 loaded PDELNs has shown significant improvement in the body weight, liver histoarchitecture and serum levels of ALT, AST and ALP when compared to the disease and free peptide groups. The results indicate that the *C. procera* derived PDELNs are an appropriate biocompatible nanocarrier for targeted treatment of HCC and are promising for improved delivery and therapeutic effects in the treatment of HCC with Polybia-MP1.

1. Introduction

Hepatocellular carcinoma (HCC) is the most prevalent type of primary liver cancer, which is responsible for about 75–85% of all primary liver tumors, and is one of the most frequent causes of cancer deaths(1). Although there have been significant advances in diagnosis and treatment, the prognosis of HCC remains poor, with most cases being diagnosed at late stages when curative interventions are no longer feasible. In selected patients, clinical outcomes have improved with the current treatment modalities such as surgical resection, liver transplantation, chemotherapy, radiotherapy, targeted therapy and immunotherapy. But they suffer from a number of general limitations, such as systemic toxicity, drug resistance, lack of tumor selectivity and low long-term survival rates(2, 3). These limitations highlight the importance of developing safer, more effective therapeutic strategies that can effectively target the tumor, while minimizing toxicity.

Peptides are receiving a lot of attention in the emerging treatments due to their high biological activity, specificity, and comparatively low toxicity. Short amino acid sequences, called anticancer peptides (ACPs), are able to selectively target cancer cells by disrupting cell membranes, inducing apoptosis, modifying pathways of cell signaling, and inhibiting angiogenesis(4). Since many cancer cell membranes are characterized by a specific lipid composition, ACPs are promising targeted cancer therapeutic agents as they are selectively toxic to the cancer cells, but not to the normal ones.

The Venom-derived peptides are a significant group of ACPs due to their strong biological activities. The venom of *Polybia paulista*, a native wasp of Brazil, is a source of a cationic antimicrobial peptide, called Polybia-MP1 (MP1), which has been shown to have selective cytotoxicity toward a variety of cancer cells, and is comparatively less toxic to normal cells. Its selectivity is thought to be due to its interaction with negatively charged phospholipids such as phosphatidylserine and phosphatidylethanolamine, which are abnormally exposed on the surfaces of the membranes of cancer cells. MP1 binding to these phospholipids causes the

membrane to become compromised, forms pores and eventually kills the cancer cell(5). These characteristics make MP1 an interesting therapeutic candidate for cancer treatment.

Although peptide-based drugs have a therapeutic potential, there are a number of challenges that limit their clinical application. Peptides undergo enzymatic hydrolysis, have poor stability and bioavailability and are quickly metabolized out of the circulation. Furthermore, they have a poor capacity to enter cells, thus decreasing their therapeutic activity(6). Thus, the design and development of effective drug delivery systems for the protection of peptides from degradation, their longer circulation time and their better intracellular delivery has been an important research field.

In this context, exosomes have received much attention as natural nanocarriers for therapeutic delivery. The nanosized extracellular vesicles are released by most cell types, usually measuring between 30 and 150nm, and play a key role in intercellular communication by transporting proteins, lipids and nucleic acids(7). Due to their biocompatibility, low immunogenicity, penetration of biological barriers, and the efficient cellular uptake, exosomes have become a promising carrier of drugs, nucleic acids and peptides. In addition, the encapsulation of therapeutic molecules in exosomes may enhance their stability, bioavailability, cellular uptake, and controlled targeted delivery and decrease the systemic toxicity(8, 9).

Although mammalian cell-derived exosomes have been extensively investigated, recently, there is mounting interest in using plant-derived exosome-like nanoparticles (PDELNs), ranging in size from 100 to 500 nm, as an alternative drug delivery platform. PDELNs share some structural and functional features with mammalian exosomes, and have several practical benefits such as large-scale production, low toxicity, high physiological stability and excellent biocompatibility(10). Their lipid nature is excellent for the uptake by cells and for protecting encapsulated therapeutic molecules from degradation and hence for potential targeted drug delivery applications.

Calotropis procera a medicinal plant that is widely used throughout the tropics and subtropics and has been known for its many medicinal properties. *C. procera* latex is rich in bioactive compounds that have been shown to have antimicrobial, antioxidant, anti-inflammatory, and anticancer properties(11, 12). Further, plant latex has also been studied as a source of exosome-like nanoparticles, which have potential biomedical applications, more recently. Yet, the application of *C. procera* derived exosome-like nanoparticles as peptide therapeutics delivery vehicles is still under-explored.

Therapeutic molecules have been loaded into exosomes through multiple approaches such as incubation, electroporation, extrusion, and sonication, as well as freeze-thaw cycling, saponin-assisted permeabilization, and genetic transfection of donor cells(13). Encapsulation within exosomes has been shown to enhance drug stability, improve cellular uptake, and provide sustained release while reducing off-target toxicity. Plant-derived exosome-like nanoparticles have been reported to have great potential to be used as vehicles to deliver drugs; however, there is still limited research exists on the delivery of Polybia-MP1 using *Calotropis procera*-derived exosome-like nanoparticles. Furthermore, they are still poorly studied for their therapeutic efficacy in in vitro and in vivo models of hepatocellular carcinoma.

Therefore, the present study aimed to explore the use of *Calotropis procera*-derived exosome-like nanoparticles as a natural nanocarrier for delivery of anticancer peptide Polybia-MP1. The isolated nanoparticles were investigated in terms of their physicochemical characteristics, peptide encapsulation efficiency, and release

profile, and then tested for their anticancer activity in HepG2 cells and a DEN/CCl₄-induced Wistar rat model of HCC. This study aims to provide insight into the application of plant-derived exosome-like nanoparticles as a biocompatible delivery platform for peptide-based therapy against HCC.

2. Materials and Methods

Materials

Fresh *Calotropis procera* latex was collected from mature plants maintained at the Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan, during morning hours in the vegetative stage, and a voucher specimen was deposited at the institutional herbarium. Polybia-MP1 peptide (> 98% purity, 1654.01 Da) was obtained in lyophilized form from a commercial supplier, and its identity was verified via electrospray ionization mass spectrometry (ESI-MS). Polyethylene glycol 6000 (PEG6000), phosphate-buffered saline (PBS), bovine serum albumin (BSA), Bradford reagent, sodium dodecyl sulfate (SDS), acrylamide/bis-acrylamide, silver staining reagents, dimethyl sulfoxide (DMSO), MTT reagent, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, diethylnitrosamine (DEN), carbon tetrachloride (CCl₄), olive oil, trehalose, and other analytical-grade chemicals were purchased from commercial suppliers and used as received. The human hepatocellular carcinoma cell line HepG2 was obtained from the Cell Culture Facility, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan; the line was authenticated using short Tandem Repeat (STR) profiling, confirmed negative for mycoplasma contamination, and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂. For Western blot analysis, Anti-TET8 primary antibody (PHY1490S, PhytoAB, San Jose, CA, USA), anti-HSP70 primary antibody (PHY0167, PhytoAB, San Jose, CA, USA), goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (ab97048, Abcam, Cambridge, UK), CDP-Star™ Chemiluminescent Substrate (T2146, Thermo Fisher Scientific, Waltham, MA, USA) were used. Nanoparticle hydrodynamic diameter size, polydispersity index (PDI), and zeta potential were measured using a Litesizer™ 500 (Anton Paar, Austria) via dynamic light scattering (DLS) at a controlled temperature of 25°C with a 175° backscatter detection angle. Morphological characterization was performed using an FEI Inspect S50 scanning electron microscope operated at an accelerating voltage of 15 kV following sample mounting on carbon tape and sputter-coating with a thin layer of gold-platinum. Quantitative RP-HPLC analysis was conducted on a Shimadzu LC-20 HPLC system equipped with a C18 reverse-phase column (5 µm, 4.6 × 250 mm) under a linear gradient of 0.1% trifluoroacetic acid (TFA) in water (Mobile Phase A) and acetonitrile (Mobile Phase B) at a constant flow rate of 1.0 ml/min, with the eluent monitored via UV detection at a peptide-optimized wavelength of 220nm.

Methods

2.1. Collection of *Calotropis procera* Latex

Fresh opaque latex was harvested from mature, healthy *Calotropis procera* plants growing at the Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan, during the morning hours to ensure optimal vesicle yield. A small incision was made on the stem and petioles using a sterile scalpel blade, and exuding latex was collected directly into pre-cooled, sterile 50mL centrifuge tubes containing a protease inhibitor cocktail to prevent proteolytic degradation. The latex was immediately diluted with an equal volume of

ice-cold phosphate-buffered saline (PBS, 1x, pH 7.4) and gently inverted to prevent coagulation and clotting. The mixture was filtered through a sterile four-layer cheesecloth to remove coarse plant debris, stored at 4°C and processed within 24 h for the downstream isolation of plant-derived exosome-like nanoparticles (PDELNs).

2.2. Isolation of Plant-Derived Exosome-Like Nanoparticles

Plant-derived exosome-like nanoparticles (PDELNs) were isolated from *Calotropis procera* latex using a polyethylene glycol (PEG)-based precipitation method with slight modifications(14). Briefly, 5 mL of fresh latex was diluted with an equal volume of phosphate-buffered saline (PBS, pH 7.4) and filtered through multi-layer sterile gauze to remove debris. The resulting filtrate was subjected to sequential centrifuged at 4°C (2,000 × *g* for 10 min, 6,000 × *g* for 20 min, and 10,000 × *g* for 40 min) to systematically remove intact cells, cellular fragments, and larger rubber particles. High-molecular weight PEG 6000 was subsequently added to the clarified supernatant to achieve a final working concentration of 8% (w/v), followed by overnight incubation at 4°C to precipitate the nanovesicles. The crude PDELNs were recovered by centrifugation at 8000 × *g* for 30 min at pellet 4°C. The supernatant was decanted, and the resulting pellet was gently resuspended in Milli-Q water. Finally, the suspension was clarified by sequential passage through 0.45 µm and 0.22 µm syringe filters, aliquoted into low-binding microcentrifuge tubes, and stored at -20°C for subsequent characterization and loading procedures until further use.

2.3. Physiochemical and Biophysical Characterization of Native and MP1-Loaded PDELNs

We evaluated how Polybia-MP1 peptide encapsulation impacts the nanovesicles by performing a side-by-side comparative analysis of empty and cargo-loaded plant derived exosome-like nanoparticles (PDELNs). Scanning electron microscopy (SEM) captured their physical morphology and membrane structural integrity under a constant accelerating voltage. Next, the biophysical dimensions of both groups were measured. Dynamic light scattering (DLS) handled the analysis here, calculating the mean hydrodynamic diameter, intensity-based size distribution profiles, and the overall polydispersity index (PDI) at a stable temperature. Concurrently, phase analysis light scattering (PALS) tracked zeta potential values to monitor for shifts in surface charge and electrokinetic stability following cargo loading. The instrument's native software processed these light scattering inputs, and we analyzed the samples in triplicate to ensure statistical reproducibility.

2.3.1. SDS-PAGE and Silver Staining

The protein profile of the isolated PDELNs was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(15). Purified PDELN samples were mixed with an equal volume of 2 X Laemmli sample loading buffer and thermally denatured at 95°C for 10 min. Equal amounts of exosomal proteins (20 µg per lane) were subsequently resolved on a discontinuous polyacrylamide gel system consisting of a 4% stacking gel and a 12% resolving gel. Electrophoretic separation was conducted at a constant voltage of 120V for 1 h 40 min in a standard Tris-glycine-SDS running buffer. Following electrophoresis, total protein bands were visualized using the highly sensitive Pierce™ Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. Gels were imaged immediately after development to analyze the relative abundance and molecular weight distribution of the PDELN associated proteins.

2.3.2. Western Blotting

Western blot analysis was performed to confirm the presence of the plant exosomal markers TETRASPANIN 8 (TET8) AND Heat Shock Protein 70 (HSP70) in the isolated PDELNs(16, 17). Protein samples resolved via 12% SDS-PAGE were electroblotted onto a 0.45 μm nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA, USA) using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding sites were blocked by incubating the membrane with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature with gentle agitation. The membrane was subsequently incubated overnight at 4°C with rabbit polyclonal anti-TET8 primary antibody (Cat. No. AS18 4182, Agrisera, Vännäs, Sweden; diluted 1:500) and rabbit polyclonal anti-HSP70 primary antibody (Cat. No. AS08 371, Agrisera, Vännäs, Sweden; diluted 1:500) prepared in blocking buffer. Following three 10-min washes with TBST, the membrane was incubated with a goat anti-rabbit IgG alkaline phosphatase (AP)-conjugated secondary antibody (Cat. No. AS09 607, Agrisera, Vännäs, Sweden; diluted 1:2,000) for 2 h at room temperature. After extensive washing with TBST, immunoreactive protein bands were developed using CDP-Star™ Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualized using an iBright™ FL1000 Imaging System (Invitrogen, Waltham, MA, USA).

2.3.3. Protein Quantification

The protein concentration of the isolated PDELNs was quantified via the Bradford colorimetric method(18) using bovine serum albumin (BSA) as the calibration standard. To determine total exosomal protein concentration (including encapsulated cargo), PDELN aliquots were lysed and denatured by incubating with 2 X SDS loading buffer at 70°C for 5 min. Concurrently, to estimate the abundance of surface-accessible proteins, intact PDELN counterparts were processed directly in the absence of thermal lysis. Absorbance profiles were recorded at 595 nm using a microplate spectrophotometer. Final protein yields were calculated through interpolation against a linear BSA standard curve.

2.3.4. Field Emission Scanning Electron Microscopy

The surface morphology and structural topography of the isolated plant-derived exosome-like nanoparticles (PDELNs) were evaluated using a field emission scanning electron microscope (FE-SEM; Regulus 8230, Hitachi High-Technologies, Tokyo, Japan). Lyophilized PDELN samples were uniformly mounted onto aluminum stubs using conductive carbon adhesive tape. To prevent surface charging and optimize secondary electron emissions, the specimens were sputter-coated with a thin, uniform layer of gold-palladium alloy (Au-Pd) utilizing a Q150R Sputter Coater (Quorum Technologies, Laughton, East Sussex, UK). High-resolution imaging was subsequently executed under high-vacuum conditions at an accelerating voltage of 15 kV.

2.3.5. Particle Size, Polydispersity Index, and Zeta Potential

The hydrodynamic diameter (particle size), polydispersity index (PDI), and zeta potential of both unloaded and MP1-loaded plant-derived exosome-like nanoparticles (PDELNs) were determined using a Litesizer™ 500 light-scattering instrument (Anton Paar GmbH, Graz, Austria). Prior to analysis, concentrated PDELN suspensions were diluted (1:1,000, v/v) in filtered Milli-Q water to avoid multiple-scattering artifacts. Particle size and PDI were characterized via dynamic light scattering (DLS) at a fixed backscattering angle of 175° to optimize sensitivity for small nanoparticles. Surface charge profiles (zeta potential) were concurrently evaluated at 25°C via electrophoretic light scattering using continuously adapted phase-amplitude light scattering (cmPALS) technology. All measurements were conducted in triplicate using dedicated Omega cuvettes, and downstream data processing was executed using Kalliope™ software (v2.2.0, Anton Paar).

2.4. Preparation and Analysis of Polybia-MP1 Peptide

Synthetic Polybia-MP1 peptide (> 98% purity) was obtained in lyophilized form. The peptide was reconstituted in ultrapure water to generate a 1 mg/mL stock solution, which was partitioned into single-use aliquots and stored at -80°C. The structural purity of the peptide was validated via analytical reverse-phase high-performance liquid chromatography (RP-HPLC) utilizing a Shimadzu LC-20 HPLC system. Chromatographic separation was achieved on a Jupiter® C18 reverse-phase column (250 mm x 4.6 mm, 5 μ m particle size, 300 Å pore size; Phenomenex, Torrance, CA, USA). Elution was executed via a linear gradient method using Mobile Phase A (0.1% trifluoroacetic acid in water) and Mobile Phase B (0.1% trifluoroacetic acid in acetonitrile) at a constant flow rate of 1.0 mL/min. Eluted peptide peaks were monitored via UV absorbance at 210 nm, and peak area integration was performed to confirm bulk purity profiles.

2.5. Peptide Loading

Polybia-MP1 peptide was loaded into the isolated plant-derived exosome-like nanoparticles (PDELNs) using an optimized sonication method. Briefly, 1 mL of purified PDELN suspension was thoroughly mixed with 100 μ L of Polybia-MP1 peptide solution (100 μ g/mL). The mixture was subjected to ultrasonication utilizing a Misonix XL-2000 Series Ultrasonic Liquid Processor (Misonix Inc., Farmingdale, NY, USA) equipped with a microtip probe. The sample was processed for 10 cycles consisting of a 6 s pulse and a 2 s pause at an operational output voltage of 20 V. To prevent potential thermal degradation of the vesicular membrane structures, this entire sonication process was repeated twice, interspersed with short cooling intervals on ice between cycles. The resulting suspension was subsequently incubated at 37°C for 1 h to facilitate membrane recovery and structural stabilization of the freshly formulated MP1-loaded PDELNs. To separate the nanoparticles from unencapsulated, free peptide molecules, the mixture was centrifuged at 13,000 rpm (approximately 16,100 x g) at 4°C for 40 min. The pellet containing the isolated MP1-loaded PDELNs was gently washed with sterile phosphate-buffered saline (PBS), centrifuged again at 13,000 rpm (approximately 16,100 x g) for 20 min, and the final sedimented nano-formulation was homogeneously resuspended in 200 μ L of PBS. Samples were stored in a dark environment at -20°C until downstream experimental utilization.

2.6. Lyophilization

Both blank (unloaded) and MP1-loaded plant-derived exosome-like nanoparticles (PDELNs) were lyophilized in the presence of trehalose as a cryoprotectant to preserve vesicular structural integrity during long-term storage(19, 20). The nano-formulation samples were flash-frozen in liquid nitrogen and subsequently transferred to a Christ Alpha 1-2 LD Plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Primary drying was conducted under high vacuum at an ice condenser temperature of (-55°C) and an operational chamber pressure of 0.12 mbar for an optimized duration of 3 h. The resulting lyophilized cake matrices were stably preserved at 4°C in a desiccator environment. Prior to downstream characterization, the dry powders were fully reconstituted back to their initial original volume using sterile, ultrapure water.

2.7. Encapsulation Efficiency

The encapsulation efficiency (EE%) of MP1-loaded PDELNs was determined by measuring the amount of unencapsulated peptide remaining after the loading process. Following peptide loading, the suspension was

centrifuged to separate free MP1 from the MP1-loaded PDELNs. The supernatant containing the unencapsulated peptide was collected, and the peptide concentration was determined by UV spectrophotometry at 280 nm. The encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation Efficiency (EE \%)} = \frac{\text{Total peptide} - \text{Free peptide}}{\text{Total peptide loaded}} \times 100$$

2.8. In Vitro Drug Release Study

The in vitro release profile of MP1 from the loaded PDELNs was evaluated using the direct dispersion method. Briefly, 100 mg of lyophilized MP1-loaded PDELNs were dispersed in 10 mL of phosphate-buffered saline (PBS) and incubated at 37°C under gentle agitation. A 1 mL aliquot was collected immediately (0 h) and subsequently at 2, 4, 6, 8, 12, and 24 h. After each sampling, an equal volume of fresh PBS was added to maintain a constant release volume and sink conditions. The collected samples were centrifuged, and the amount of released MP1 in the supernatant was determined by UV spectrophotometry at 280 nm. The cumulative release of MP1 was calculated using the following equation:

$$\text{Cumulative release (\%)} = \frac{(C_t \times V) + \sum_{i=1}^{t-1} (C_i \times V_s)}{\text{Initial amount of peptide loaded}} \times 100$$

Where C_t is the peptide concentration at the current sampling time, C_i is the peptide concentration at each previous sampling time, V is the total release volume, and V_s is the sample volume withdrawn at each time point.

2.9. In Vitro Cytotoxicity Assay

The cytotoxic effects of free MP1 peptide, unloaded PDELNs, and MP1-loaded PDELNs were evaluated using the MTT assay⁽²¹⁾ on HepG2 human hepatocellular carcinoma cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After reaching the required growth, the cell suspension was adjusted to 5×10^5 cells/mL, and 100 µL was seeded into each well of a 96-well plate (5×10^3 cells/well). The cells were incubated for 24 h before treatment.

Free MP1 peptide, unloaded PDELNs, and MP1-loaded PDELNs were prepared in DMEM supplemented with 10% FBS. Serial dilutions were made to obtain final concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL. Untreated cells and doxorubicin-treated cells served as the negative and positive controls, respectively. All treatments were performed in triplicate, and the plates were incubated for 48 h.

Following treatment, the culture medium was replaced with fresh medium containing 10 µL of MTT solution (5 mg/mL) and incubated for 3 h. The medium was then removed, and the resulting formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader, and cell viability was calculated relative to the untreated control.

$$\text{Survival Rate (\%)}_{570 \text{ nm}} = \frac{\text{Absorbance of Sample} - \text{Absorbance of the Blank}}{\text{Absorbance of Positive Control} - \text{Absorbance of the Blank}} \times 100$$

2.10. In Vivo Anticancer Study

2.10.1. Experimental Animals

Fifteen healthy male Wistar albino rats (100–130 g, 6–7 weeks old) were obtained from the animal facility of the Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore. The animals were housed under standard laboratory conditions ($22 \pm 2^\circ\text{C}$, 50–60% relative humidity, and a 12 h light/dark cycle) with free access to a standard pellet diet and water. All experimental procedures were approved by the Institutional Animal Ethics Committee of CEMB, University of the Punjab.

2.10.2. Experimental Design

The rats were randomly divided into a normal control group ($n = 2$) and an experimental group ($n = 13$). The normal control group received 0.25% normal saline (1 mL/kg), whereas hepatocellular carcinoma (HCC) was induced in the experimental group by intraperitoneal administration of diethylnitrosamine (DEN; 100 mg/kg) and carbon tetrachloride (CCl_4 ; 0.25 mL/kg, prepared as a 10% v/v solution in olive oil) twice weekly for 8 weeks(22).

After 8 weeks, one animal from the experimental group was sacrificed to confirm HCC induction by histopathological examination of liver tissue. Following confirmation, the remaining animals were randomly assigned to four treatment groups ($n = 3$ per group): disease control, free MP1 peptide (0.2 mg/kg), MP1-loaded PDELNs (5 mg/kg), and unloaded PDELNs (5 mg/kg). Treatments were administered intraperitoneally twice weekly for 6 weeks. Animals were monitored daily for general health and behavioral changes throughout the study.

2.10.3. Biochemical Analysis

At the conclusion of the treatment period, the rats were deeply anesthetized via intraperitoneal injection of ketamine (80-100mg/kg) and xylazine (10mg/kg) and whole blood samples were collected via terminal cardiac puncture. To isolate the serum, the collected blood was allowed to clot at room temperature for 30 min, followed by centrifugation at 4,000 rpm (approximately $2,000 \times g$) for 10 min at 4°C . The resulting supernatant serum was carefully harvested, partitioned into aliquots, and cryopreserved at -20°C until further analysis. To evaluate potential systemic hepatotoxicity, the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were quantified using commercially available diagnostic kits (Human Diagnostic Worldwide (Wiesbaden, Germany) on an automated clinical biochemistry analyzer Humalyzer 3000(Human Diagnostics, Wiesbaden, Germany) according to the manufacturers' protocols

2.10.4. Histopathological Evaluation and Organ Index Analysis

Following humane sacrifice, liver tissues were immediately excised, thoroughly rinsed with ice-cold sterile saline, and macroscopically examined. For histopathological evaluation, the tissue specimens were fixed in 10% (v/v) neutral buffered formalin for 24 h, dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin wax blocks. Serial tissue sections with a thickness of $5 \mu\text{m}$ were prepared using a rotary microtome, mounted onto glass slides, and subjected to standard hematoxylin and eosin (H&E) staining. Morphological alterations, hepatic damage scores, and therapeutic responses within the hepatocellular carcinoma (HCC) foci were examined and imaged under a high-resolution brightfield microscope. To track systemic physiological impacts, individual animal body weights were recorded at the baseline initiation and the

experimental endpoint conclusion. Liver weights were measured immediately post-mortem to determine the hepatosomatic index (HSI), computed via the following equation:

Hepatosomatic Index (HSI) = ($\frac{\text{Liver Weight (g)}}{\text{Final Body Weight (g)}}$) x 100

2.11. Statistical Analysis

All experimental data are expressed as the mean ± standard deviation (SD) of at least three independent replicates (n ≥ 3). Statistical evaluations were performed utilizing GraphPad Prism software (version 10.4.2; GraphPad Software Inc., San Diego, CA, USA). Data normality and homogeneity of variance were systematically verified using the Shapiro-Wilk and Brown-Forsythe tests, respectively, where applicable; given the small group sizes in the in vivo experiments (n = 2–3 group), normality assumptions could not be robustly assessed, and results from these comparisons should be interpreted with appropriate caution. Comparative assessments among multiple experimental cohorts were executed via one-way or two-way analysis of variance (ANOVA), followed by *post hoc* Tukey’s honestly significant difference (HSD) multiple comparisons test. A probability value of p < 0.05 was established as the threshold for statistical significance.

3. Results

3.1. Isolation and Characterization of Plant-Derived Exosome-Like Nanoparticles

Nanoparticle fractions resembling plant-derived exosome-like vesicles (PDELNs) were efficiently isolated from *Calotropis procera* latex using a polyethylene glycol (PEG)-mediated precipitation approach. To validate successful recovery and assess the physical impact of peptide encapsulation, the biophysical profiles of both pristine (Free) PDELNs and MP1-loaded formulations were systematically characterized (Table 1). The un-complexed Free PDELNs exhibited an average hydrodynamic diameter of 187.7 nm with a polydispersity index (PDI) of 0.545, alongside a net negative surface charge of − 10.3 ± 76.9 mV. Following the incorporation of the Polybia-MP1 peptide, a distinct rightward shift in particle size to 251.4 nm was recorded, signaling successful cargo loading and structural expansion of the vesicle matrices. Interestingly, peptide loading was accompanied by a reduction in the PDI to 0.334, indicating a more homogeneous size distribution post-formulation. Concurrently, the surface charge experienced a marginal electrokinetic transition to − 8.33 ± 56.4 mV (Table 1). Combined with total protein profiling and morphological imaging, these DLS trends confirm the baseline structural stability of the isolated vesicle platforms and their capacity to adapt to active therapeutic cargo overloading without triggering systemic macro-aggregation.

Table 1
Dynamic light scattering (DLS) analysis

Sample	Diameter (nm)	PDI (-)	Zeta Potential (mV)
Free PDELNs	187.7	0.545	-10.3 ± 76.9
MP1-loaded Exosomes	251.4	0.334	-8.33 ± 56.4

3.1.1. Protein Profile of PDELNs and Western Blot Analysis

The protein composition of the isolated *Calotropis procera* fractions was resolved via SDS-PAGE and visualized through high-sensitivity silver staining (Fig. 1A). The electrophoretic separation revealed a clear distinction between the proteomic signatures of chemically lysed and intact nanoparticle matrices. In the intact PDELN lanes, a restricted and highly specific distribution of outer-surface protein bands was resolved, characterized by distinct bands migrating near 10 kDa and spanning the 27 to 38 kDa range (indicated by directional arrows). Conversely, the lysed PDELN samples displayed a dense, continuous profile of multi-weight bands, reflecting a richly diverse intravesicular proteome securely encapsulated within the protective boundaries of the native lipid bilayer framework.

To confirm the authentic endosome-derived vesicular lineage of these isolated platforms, Western blot analysis was performed (Fig. 1B). The chemiluminescent immunoblots demonstrated highly specific, distinct immunoreactive bands corresponding to the essential plant extracellular vesicle membrane scaffold marker, TETRASPANIN 8 (TET8), and the intravesicular molecular chaperone, Heat Shock Protein 70 (HSP70). The simultaneous detection of these two benchmark exosomal markers definitively validates the successful recovery, high purity, and biochemical integrity of the isolated PDELN platforms.

3.1.2. Protein Quantification and Morphological Analysis

To evaluate the distribution of vesicular protein cargo, a colorimetric Bradford assay was performed to compare intact and thermally lysed nano-formulations. The surface-accessible protein concentration of the intact PDELNs was determined to be 0.130 mg/ml. Upon chemical and thermal lysis, the total detectable protein concentration significantly increased to 0.230 mg/ml. This distinct, near-twofold elevation in protein yield within the lysed cohorts directly confirms that a substantial proportion of the plant proteomic matrix is securely encapsulated and protected inside the internal core of the vesicles.

Following quantitative protein analysis, the structural architecture and surface topography of the isolated nanoparticles were examined via high-resolution scanning electron microscopy (Fig. 1C). The resulting electron micrographs revealed a highly dense and uniform population of intact nanostructures. Under high-vacuum conditions, the isolated PDELNs exhibited a predominantly spherical to near-spherical geometric symmetry characterized by completely smooth, continuous outer surface boundaries. No signs of structural deflation, lipid bilayer fissuring, or random macro-aggregation were observed, confirming that the processing conditions effectively preserved the native three-dimensional morphology of the nanoparticle platforms.

3.1.3. Particle Size, Polydispersity Index, and Zeta Potential

To verify successful drug incorporation and assess colloidal dynamics in solution, dynamic light scattering (DLS) and electrophoretic light scattering were executed on both pristine and formulated nanocarriers (Table 1). The isolated baseline PDELNs exhibited a mean hydrodynamic particle size of 187.7 nm coupled with a polydispersity index (PDI) of 0.545. Following the structural encapsulation of the Polybia-MP1 peptide, the average hydrodynamic diameter shifted rightward to 251.4 nm. This noticeable volumetric expansion provides direct physical validation of successful intravesicular cargo loading within the nanoparticle core, aligning with the spherical dimensions tracked via microscopy (Fig. 1C). Interestingly, peptide encapsulation triggered a concurrent decline in the PDI to 0.334, indicating that the nano-formulation transitioned toward a significantly more uniform and stable monodisperse population post-sonication. Electrokinetic analysis revealed that the native, unloaded PDELNs possessed a negative zeta potential of -10.3 ± 76.9 mV, which subsequently

modulated to -8.33 ± 56.4 mV upon active peptide loading (Table 1). This electrical neutralization confirms that the cationic Polybia-MP1 peptide successfully interacted with and integrated into the negatively charged lipid framework of the vesicles, significantly altering the surface properties of the resulting nano-formulations to facilitate sustained cargo retention (Table 1).

3.2. Characterization of Polybia-MP1 Peptide

Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed to verify the chemical homogeneity and baseline purity of the synthetic Polybia-MP1 peptide prior to its formulation with the vesicular nanocarriers (Fig. 2). Spectrophotometric monitoring at a detection wavelength of 220 nm revealed a highly stable baseline throughout the elution timeline, featuring a single, exceptionally sharp and symmetrical dominant peak. This major peak exhibited an exact retention time of 31.00 min, which corresponds precisely to the target therapeutic peptide matrix. Quantitative peak area integration established that the bulk chemical purity of the peptide was approximately 98%, with negligible secondary peak noise or structural degradation fractions. These chromatographic profiling results confirm that the Polybia-MP1 peptide possesses high chemical purity and structural integrity, validating its suitability for subsequent stable intravesicular encapsulation and downstream biological evaluations.

3.2.1. Evaluation of the Ultrasonication Peptide Loading Process

To establish an optimal nano-formulation protocol, the incorporation of the Polybia-MP1 peptide into the plant-derived exosome-like nanoparticles (PDELNs) was systematically monitored during the probe-sonication sequence. The application of 10 ultrasonic pulse cycles at an operational voltage of 20 V, executed in duplicate with intermediate cooling intervals on ice, successfully facilitated cargo internalization without inducing macroscopic lipid precipitation or protein denaturation. Post-sonication, the colloidal suspension remained completely translucent and free of visible particulate aggregates, demonstrating that the vesicular membranes safely tolerated the mechanical and thermal stresses of the loading process. This procedural success was subsequently validated by the distinct physical size shifts quantified during downstream solution profiling.

3.2.2. Solid-State Stability and Reconstitution Efficiency of Lyophilized PDELNs

To ensure long-term benchtop preservation, both blank and MP1-loaded PDELNs underwent structural stabilization via vacuum freeze-drying using a Christ Alpha 1–2 LD Plus lyophilizer. In the presence of trehalose as a lyoprotectant matrix, the 3 h accelerated dehydration cycle consistently yielded a highly elegant, uniform, and fluffy off-white lyophilized cake with zero signs of structural collapse, melting, or hygroscopic shrinking at the bottom of the vials. Crucially, upon the addition of the original volume of ultrapure water, the dry cake matrix exhibited exceptional reconstitution kinetics, dissolving completely in under 30 seconds with gentle agitation. The reconstituted liquid fully restored its native, slightly opalescent colloidal appearance without generating precipitate flakes or altering baseline solution dynamics, confirming that the optimized lyophilization protocol effectively locked the structural configuration of the nanoparticles in the solid state.

3.3. Encapsulation Efficiency and In Vitro Drug Release

To evaluate the payload capacity and subsequent delivery dynamics of the developed nano-formulation, the encapsulation efficiency (EE%) and cumulative kinetic release profiles of the Polybia-MP1 peptide from the nano-vesicles were quantified. Quantitative analysis of the post-centrifugation supernatant established that the EE% of MP1 within the plant-derived exosome-like nanoparticles (PDELNs) was $42.1 \pm 2.13\%$, confirming a robust and successful peptide loading capacity into the vesicular core.

Following encapsulation validation, the *in vitro* elution behavior under simulated physiological conditions revealed a controlled, biphasic sustained release pathway over a 24 h timeline (Fig. 1D). The formulation exhibited a moderate, controlled initial release phase, with 23.9% cumulative peptide liberation recorded within the first 4 h. This was followed by a highly gradual, steady diffusion phase that reached 48.37% cumulative release at the 24 h endpoint (Fig. 1D). The absence of an immediate burst release effect indicates that the structural integrity of the PDELN lipid bilayer effectively preserves the therapeutic peptide cargo, confirming its functional utility as a sustained-delivery nanocarrier system.

3.4. In Vitro Cytotoxicity of MP1-Loaded PDELNs

Unloaded PDELNs, free MP1 peptide, and MP1-loaded PDELNs were evaluated for the cytotoxic activity against HepG2 cells using the MTT assay (Fig. 3-A). Unloaded PDELNs showed minimal cytotoxicity across the tested concentration range (1.56–100 $\mu\text{g/mL}$), maintaining cell viability above **86%** even at the highest concentration. However, both free MP1 and MP1-loaded PDELNs produced a concentration-dependent reduction in cell viability. When cells were treated with lower concentrations (1.56–6.25 $\mu\text{g/mL}$), cell viability remained above **72%** for all treatment groups. As the concentration increased, MP1-loaded PDELNs demonstrated significantly greater cytotoxicity than free MP1, resulting in a more pronounced reduction in cell viability. At **100 $\mu\text{g/mL}$** , MP1-loaded PDELNs reduced cell viability to approximately **20.03%**, whereas unloaded PDELNs maintained a cell viability of approximately **86.67%**.

The enhanced cytotoxic activity of MP1-loaded PDELNs was further confirmed by IC_{50} analysis (Fig. 3-B). The IC_{50} value of MP1-loaded PDELNs was 21.313 $\mu\text{g/mL}$, which was markedly lower than that of free MP1 (42.607 $\mu\text{g/mL}$), indicating significantly improved anticancer activity following peptide encapsulation.

3.5. In Vivo Anticancer Activity of MP1-Loaded PDELNs

3.5.1. Physiological Parameters

The systemic physiological impacts of the various therapeutic regimens on rat body weight kinetics and absolute liver masses are documented in Table 2. The healthy control cohort exhibited a steady, robust body mass gain over the experimental timeline (58.00 ± 10.61 g). In stark contrast, the untreated hepatocellular carcinoma (HCC) disease control group experienced a severe arrest in proliferation kinetics, yielding a net weight gain of only 10.00 ± 7.69 g, which is a classical hallmark of cancer-induced cachexia. Intervention with the free MP1 peptide partially alleviated this metabolic stagnation, increasing the weight gain to 23.00 ± 6.23 g. Remarkably, administration of the MP1-loaded exosomes generated the most pronounced physiological recovery among all interventional groups, driving the net body weight gain up to 28.00 ± 7.95 g and signalling a strong mitigation of systemic tumor burden.

Table 2

Effect of MP1 and MP1-loaded exosomes treatment on body and liver weight as compared to control and disease control group.

Sr. No	Groups	Initial day body weight (g)	Final day body weight (g)	Weight difference (g)	Weight of liver (g)
1	Control (n = 2)	109.00 ± 7.34	167.00 ± 7.66	58.00 ± 10.61	8.45 ± 2.38
2	Disease control	116.00 ± 4.17	126.00 ± 6.46	10.00 ± 7.69	14.15 ± 1.62
3	Free MP1	112.00 ± 3.83	135.00 ± 4.91	23.00 ± 6.23	10.52 ± 1.76
4	MP1-loaded Exosomes	117.00 ± 4.45	145.00 ± 6.58	28.00 ± 7.95	9.92 ± 1.32
5	Unloaded PDELNs	108.00 ± 8.22	119.00 ± 5.48	11.00 ± 9.88	12.54 ± 2.01

Mirroring the body mass dynamics, profound pathomorphological variations were observed in the absolute liver weights (Table 2). The untreated HCC disease control group developed severe tumor-induced hepatomegaly, with absolute liver masses escalating sharply to 14.15 ± 1.62 g compared to the healthy control baseline of 8.45 ± 2.38 g. Therapeutic administration of the MP1-loaded exosomes successfully suppressed this malignant organ hypertrophy, restricting the final liver weight to 9.92 ± 1.32 g. This therapeutic reduction significantly outperformed the results achieved by the free MP1 peptide counterpart (10.52 ± 1.76 g). Conversely, animals treated with unloaded PDELNs showed only a marginal reduction in liver mass (12.54 ± 2.01 g) and a severely compromised body weight gain (11.00 ± 9.88 g), confirming that the observed reversal of tumor-associated hepatomegaly is driven specifically by the targeted delivery of the encapsulated peptide rather than the carrier matrix alone.

3.5.2. Histopathological Evaluation

Histopathological examination of H&E-stained liver sections revealed marked differences among the experimental groups (Fig. 4). Liver tissues from the normal control group exhibited intact hepatic architecture with well-organized hepatic cords and normal hepatocytes. In contrast, the disease control group showed extensive hepatocellular damage characterized by disrupted hepatic architecture, cellular atypia, necrosis, and early fibrotic changes.

Treatment with free MP1 resulted in partial restoration of hepatic architecture with reduced cellular degeneration. The MP1-loaded PDELNs group demonstrated the most pronounced histological improvement, showing near-normal hepatic architecture with minimal necrosis and reduced cellular atypia. Animals treated with unloaded PDELNs exhibited only limited restoration, with persistent structural abnormalities remaining evident.

3.5.3. Effect of MP1-Loaded PDELNs on Serum Liver Enzymes

Serum levels of ALP, ALT, and AST were determined to evaluate the effect of treatment on liver function (Fig. 5).

Alkaline Phosphatase (ALP) Profiles

The untreated hepatocellular carcinoma (HCC) disease control group exhibited a profound, pathological elevation in serum ALP levels (206.9 ± 4.765 IU/L) compared to the healthy normal control baseline (54.03 ± 1.604 IU/L; **** $p < 0.0001$). This massive surge confirms severe biliary architecture damage and hepatocellular injury. Interventional therapy with the free Polybia-MP1 peptide significantly attenuated this trauma, reducing ALP levels to 166.3 ± 3.790 IU/L (### $p < 0.001$). Remarkably, administration of the MP1-loaded PDELNs demonstrated superior therapeutic efficacy, engineering the most pronounced reduction down to 149.3 ± 2.272 IU/L (#### $p < 0.0001$). In contrast, cohorts treated with empty, unloaded PDELNs yielded only a negligible decrease (195.8 ± 4.451 IU/L; # $p \leq 0.05$).

Alanine Aminotransferase (ALT)

Mirroring the ALP trends, serum ALT leaks were markedly elevated in the HCC disease control group (129.0 ± 3.270 IU/L) relative to the normal control baseline (52.90 ± 1.212 IU/L; **** $p < 0.0001$), signifying severe hepatocyte membrane damage. Treatment with the free MP1 peptide restricted ALT levels to 93.40 ± 1.200 IU/L (## $p < 0.01$). Crucially, encapsulation within the plant vesicular framework provided an enhanced therapeutic cushion, with the MP1-loaded PDELNs driving a further, highly significant suppression of ALT levels down to 83.80 ± 1.400 IU/L (#### $p < 0.0001$). Unloaded PDELN baseline controls exerted limited corrective effects, with values remaining high at 116.8 ± 5.518 IU/L (#### $p < 0.0001$ versus disease control).

Aspartate Aminotransferase (AST)

A statistically identical pathopharmacological pattern was observed for serum AST levels. The untreated disease control cohort displayed a distinct increase in AST concentrations (140.9 ± 1.450 IU/L) compared with the healthy control baseline (91.80 ± 4.651 IU/L; **** $p < 0.0001$). Systemic administration of the free MP1 peptide lowered circulating AST levels moderately to 129.5 ± 0.862 IU/L (## $p < 0.01$). This therapeutic recovery was optimized by the MP1-loaded PDELN nano-formulation, which successfully minimized enzyme leakage down to 124.0 ± 3.493 IU/L (### $p < 0.001$). Conversely, monotherapy with the unloaded PDELNs showed little protective value, producing only a marginal reduction to 136.7 ± 1.100 IU/L.

4. Discussion

Hepatocellular carcinoma (HCC), is one of the most common causes of cancer-related deaths globally and treatment is frequently limited by low drug bioavailability, systemic toxicity and targeting inefficiency. The present study demonstrated the successful encapsulation of Polybia-MP1 peptide into the plant derived exosome like nanoparticles (PDELNs) derived from plant *Calotropis procera* for enhancing its therapeutic efficacy against HCC. The results showed successful peptide encapsulation, extended release of the drug in vitro, increased in vitro cytotoxicity, and therapeutic efficacy in a DEN/ CCl_4 induced rat model, suggesting the potential of peptide delivery using PDELNs as a natural nanocarrier.

Due to their biocompatibility, low immunogenicity, and ease of isolation, as well as their capacity to carry a diverse array of bioactive molecules, plant-derived exosome-like nanoparticles are receiving growing interest as drug delivery systems. All these benefits render them appealing alternatives to synthetic nanocarriers,

especially for peptide therapeutics like Polybia-MP1 which often have a short half-life and limited stability in clinical settings.

PDELNs have been successfully isolated and characterized from *Calotropis procera*, thus proving to be a drug delivery platform. The isolated vesicles showed characteristic properties typical of exosome-like plant derived nanoparticles (nanoparticles, spherical morphology and a unique protein profile). Features similar to those described here for the exosome-like nanoparticles derived from plants have been reported for exosome-like nanoparticles from other plants, which further validate the isolation method used in this work(23–25). Furthermore, for extracellular vesicle characterization, we used several complementary characterization techniques such as SDS-PAGE, Western blotting, SEM, and DLS, which is in accordance with the current recommendations for EVs characterization(26, 27).

In this work, we have used the PEG based precipitation method, which was simple, economical and scalable to isolate PDELN. Distinct protein bands were found to be present in this expected range by SDS-PAGE and Western blot analysis of the isolated vesicles revealed the presence of a plant exosomal protein marker TET8 and another protein, HSP70, confirming the exosome-like identity of the isolated vesicles. Bradford assay revealed that the protein concentration of the lysed vesicles was higher than the intact vesicles, showing the presence of biomolecular cargo within the vesicles and the integrity of the vesicles. SEM also revealed mostly spherical vesicles with smooth surfaces whereas DLS revealed an average particle size of ~ 187 nm, within the reported particle size range of plant-derived exosome-like nanoparticles(28).

The successful encapsulation of Polybia-MP1 peptide was accompanied by the increase of the particle size (from ~ 187 to ~ 251 nm) and the shift in the zeta potential (from -10.3 ± 76.9 mV to -8.33 ± 56.4 mV). The changes show successful incorporation of peptides and modification of the surface of the vesicle after encapsulation. The changes in the particle size and surface charge also occurred upon cargo loading in other exosome-based delivery systems, supporting the successful loading of MP1 in the isolated PDELNs.

The purity of Polybia-MP1 peptide was checked using reverse-phase high-performance liquid chromatography (RP-HPLC) prior to its encapsulation. The chromatogram revealed a single and sharp peak at a retention time of ~ 31 min, corresponding to a high purity peptide that was suitable for further loading to PDELNs.

The efficiency of cargo loading is one of the important factors for the therapeutic potential of exosome-based drug delivery systems. From the variety of loading strategies that have been used, sonication is reported as one of the most effective strategies for loading exosome with proteins and peptides, which involves temporary destabilizing of the lipid bilayer and subsequent entry of protein and peptide cargo(29). With this technique, it was possible to encapsulate successfully the Polybia-MP1, with $42.1 \pm 2.13\%$ encapsulation efficiency.

The in-vitro release study also confirmed the successful loading of peptides, with a sustained release of up to 24 hrs. The cumulative release for an initial release was found to be at 23.9% at 4 h and continued to increase gradually to 48.37% at 24 h. An important property of a drug delivery system is sustained release which can maintain the therapeutic exposure for a longer duration and decrease the frequency of administration. Peptide-based delivery systems using exosomes have also been found to have similar sustained release properties, indicating their promising use to increase peptide stability and therapeutic efficacy. Overall, the results indicate that it is possible to encapsulate and slowly release Polybia-MP1 using PDELNs derived from *Calotropis procera*, which makes these nanocarriers a natural alternative for peptide delivery.

In vitro cytotoxicity studies also demonstrated the enhanced anticancer activity of MP1-loaded PDELNs. Unloaded PDELNs exhibited very low toxicity in HepG2 cells, suggesting good biocompatibility and that the anticancer effects observed were mainly attributed to the encapsulated MP1 peptide. The IC₅₀ of MP1-loaded PDELNs (21.313 µg/mL) was significantly lower than that of free MP1 (42.607 µg/mL) indicating that the cytotoxic efficacy of MP1 was significantly improved in the former. This increased activity may be attributed to the increased cellular uptake, protection from degradation of the peptide and prolonged release in the cells after exosomal delivery.

These results are similar to previous studies that have shown that exosome-based drug delivery improves the therapeutic effectiveness of anti-cancer drugs in HCC. Liang et al. (2021) reported that compared to the free drug, the proliferation of HepG2 cells was inhibited more by exosomes derived from mesenchymal stem cells loaded with nor-canthalidin(30). Likewise, Ye et al. (2024) showed that exosome-luteolin nanoparticles had better anticancer activity than free luteolin in the HepG2 cell line(31). These studies support the ability of exosome-based delivery systems to improve intracellular drug delivery and therapeutic response.

In vivo results also supported the superior in vitro results of the MP1 loaded PDELNs. MP1-loaded PDELNs significantly enhanced the physiological, histopathological and biochemical parameters when compared to free MP1 and unloaded PDELNs in the hepatocellular carcinoma model of DEN/CCl₄. Diseased animals had lower body weight gain, higher liver size, extensive liver damage and significantly higher liver enzyme levels in the serum. On the other hand, animals receiving MP1 containing PDELNs had significant improvement in liver architecture, significant weight recovery and liver weight was brought back close to normal.

The therapeutic efficacy of the nano-formulation was also confirmed by histopathological analysis. Disease control group showed widespread hepatocellular damage, including necrosis, cellular atypia, and early fibrotic changes. Treatment with MP1-loaded PDELNs showed a significant improvement in the restoration of hepatic architecture, with minimal necrosis and decreased cellular atypia. Free MP1 also improved tissue morphology but to a lesser extent, whereas unloaded PDELNs produced only limited histological recovery. These observations suggest that encapsulation enhanced the therapeutic effect of MP1 in vivo.

The biochemical findings were in agreement with the histopathological observations. MP1-loaded PDELNs produced a greater reduction in serum ALP, ALT, and AST levels than free MP1, indicating improved protection against hepatocellular injury. Similar improvements in liver function markers and histological recovery have been reported following treatment with exosome-based formulations in experimental HCC models(32). The enhanced therapeutic efficacy observed in the present study may be attributed to improved stability and bioavailability of MP1 following encapsulation, together with more efficient delivery of the peptide to the target tissue. To the best of our knowledge, this is the first study to demonstrate the therapeutic potential of *Calotropis procera*-derived PDELNs for the delivery of Polybia-MP1 peptide against hepatocellular carcinoma in both in vitro and in vivo models.

Interestingly, the unloaded PDELNs also gave a moderate increase in several parameters with a much lower percentage increase when compared with the effect of the MP1 loaded PDELNs. This finding is consistent with earlier reports of intrinsic biological activity of plant-based exosome-like nanoparticles such as anti-inflammatory and antioxidant effects(33). Therefore, the enhanced therapeutic outcome observed with MP1-

loaded PDELNs is likely the result of both efficient peptide delivery and the inherent biological properties of the exosomal carrier.

The enhanced therapeutic efficacy of MP1-loaded PDELNs is likely attributed to several factors. Polybia-MP1 selectively interacts with negatively charged cancer cell membranes, while exosome-mediated delivery improves peptide stability, cellular uptake, and intracellular transport. In addition, the sustained release profile observed in this study may contribute to prolonged therapeutic activity. Beyond their role as drug carriers, exosomes have also been reported to influence the tumor microenvironment through immunomodulatory and anti-inflammatory effects, which may further enhance therapeutic outcomes. Similar improvements in targeting efficiency and reduction of off-target effects have been reported for exosome-mediated delivery of therapeutic agents, including siRNA and chemotherapeutic drugs(34). Likewise, plant-derived exosome-like nanoparticles have shown intrinsic anticancer properties and the ability to inhibit tumor progression(35). To the best of our knowledge, this is the first study to investigate *Calotropis procera*-derived PDELNs as carriers for Polybia-MP1 peptide against hepatocellular carcinoma in both in vitro and in vivo models.

Despite these promising findings, the present study has several limitations. The in vivo experiments were conducted using a relatively small sample size, including a normal control group of $n = 2$, which limits the statistical power of normality testing and group comparisons and additional molecular investigations, including apoptosis markers, oxidative stress, signaling pathways, biodistribution, and pharmacokinetic analyses, were beyond the scope of this work. Future studies should explore these mechanisms in greater detail and evaluate surface engineering strategies to further improve the targeting efficiency and therapeutic performance of PDELNs.

5. Conclusion

This study shows the plant-derived exosome-like nanoparticles (derived from *Calotropis procera*) loaded with Polybia-MP1 peptide can markedly boost its anticancer effect on hepatocellular carcinoma. MP1-loaded PDELNs showed continuous release of peptide, enhanced in vitro cytotoxicity and better therapeutic efficacy in a DEN/ CCl_4 -induced rat model than free peptide. These results show that plant-derived exosome-like nanoparticles are a promising biocompatible and efficient platform for delivering peptides and further developing towards targeted nanocarriers for HCC treatment.

Declarations

Data Availability

Data will be made available on request. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Funding Declaration

This study did not get any fundings from internal or external source

Ethics Declaration

Study was conducted under approval from the ethical committee of university of the Punjab. The mice model which were used in the study handled by professionals.

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Declaration of interest statement

The authors report there are no competing interests to declare

Declaration of generative AI use

The authors report generative AI was not used in their research or preparation of this manuscript

Author Contributions

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Figures

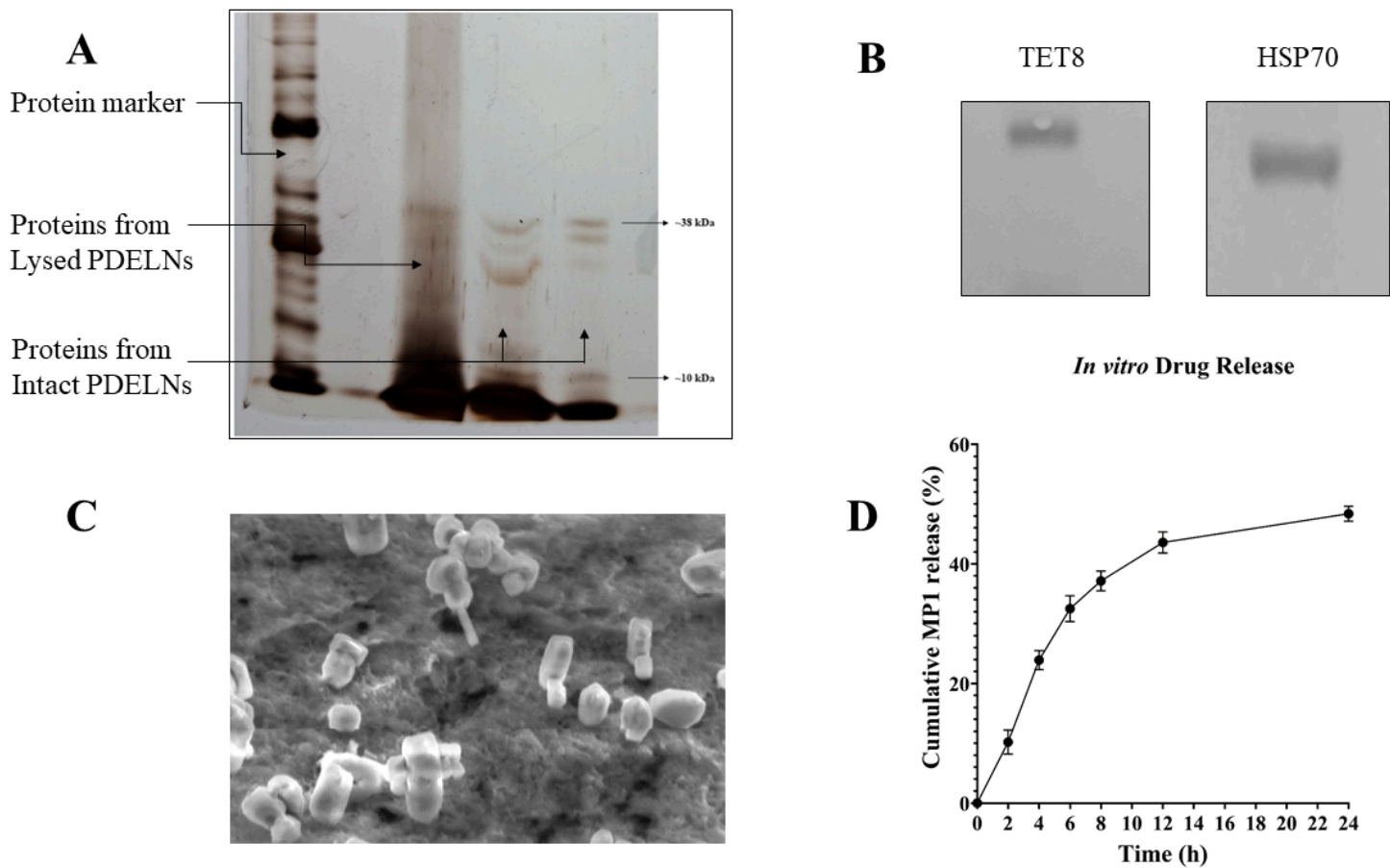


Figure 1

Characterization of plant-derived exosome-like nanoparticles (PDELNs) isolated from *Calotropis procera*. (A) SDS-PAGE analysis followed by silver staining showing the protein profile of isolated PDELNs. (B) Western blot analysis confirming the presence of exosomal marker proteins TET8 and HSP70. (C) Scanning electron microscopy (SEM) image showing the morphology of isolated PDELNs. (D) In vitro release profile of Polybia-MP1 from MP1-loaded PDELNs over 24 h.

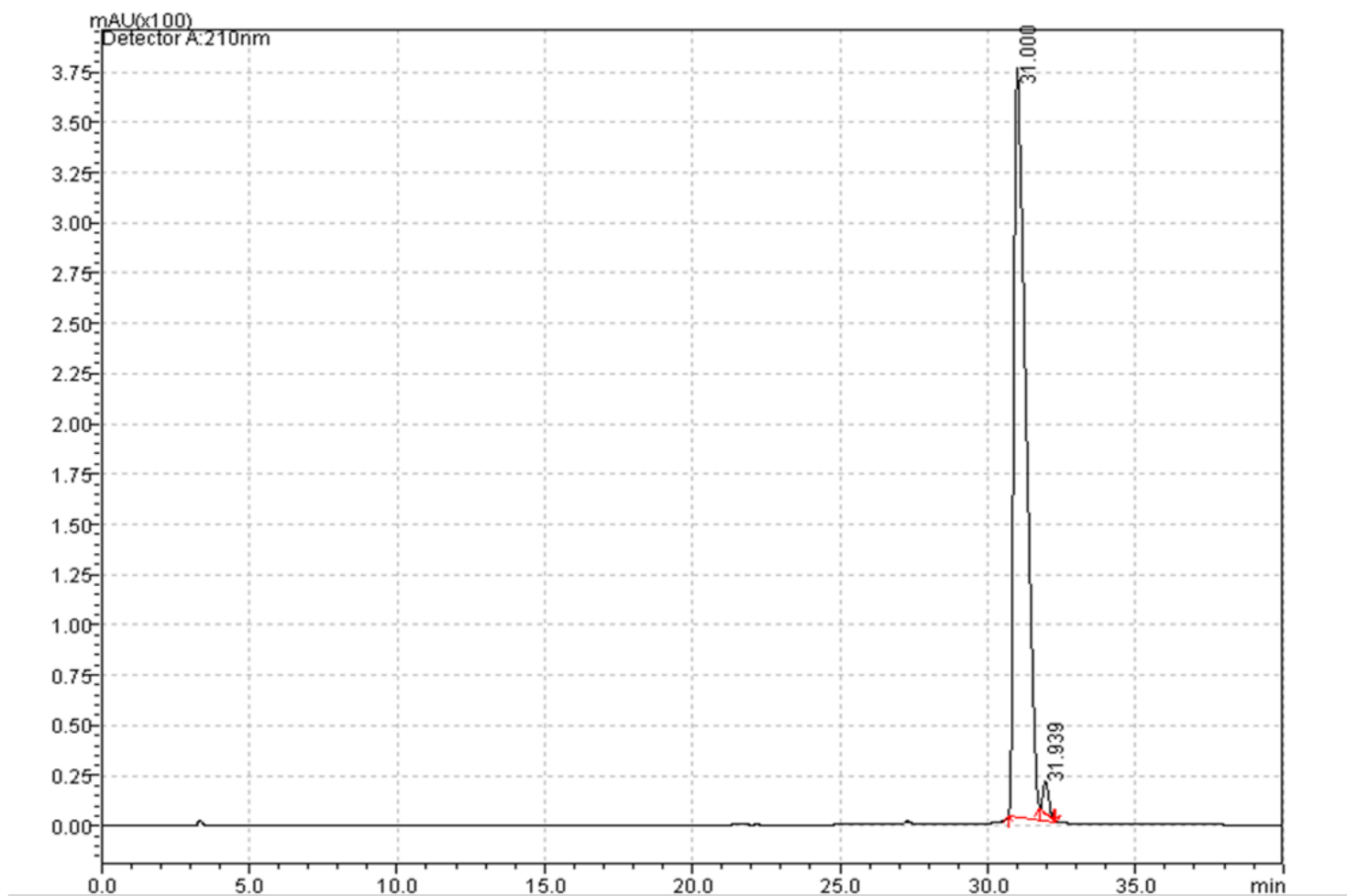
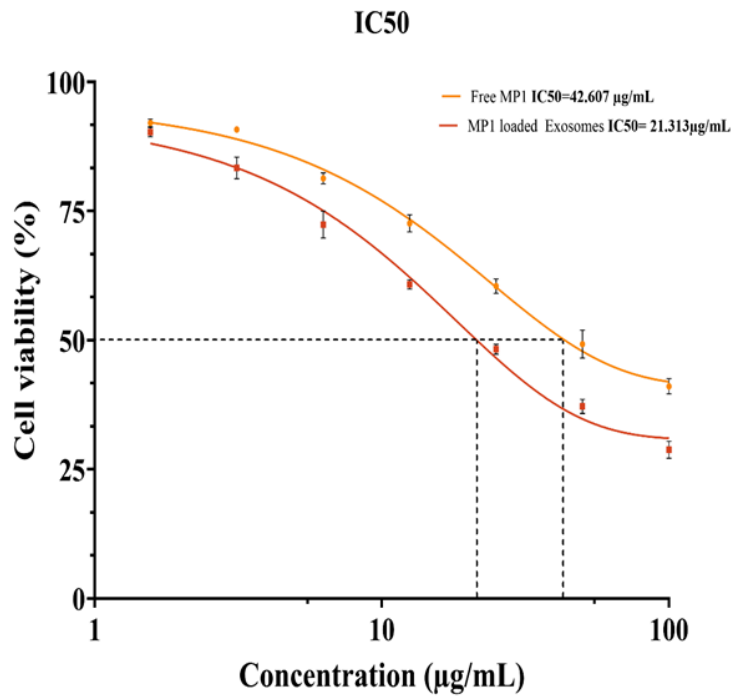


Figure 2

RP-HPLC analysis of MP1 peptide: chromatogram indicating the retention time of 31 min.

B



In vitro cytotoxicity of free MP1, unloaded PDELNs, and MP1-loaded PDELNs against HepG2 cells. (A) Cell viability of HepG2 cells following treatment with free MP1, unloaded PDELNs, and MP1-loaded PDELNs at different concentrations, as determined by the MTT assay. (B) Dose–response curves showing the IC₅₀ values of free MP1 and MP1-loaded PDELNs. One-way ANOVA: *P ≤ 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 and ns=non-significant.

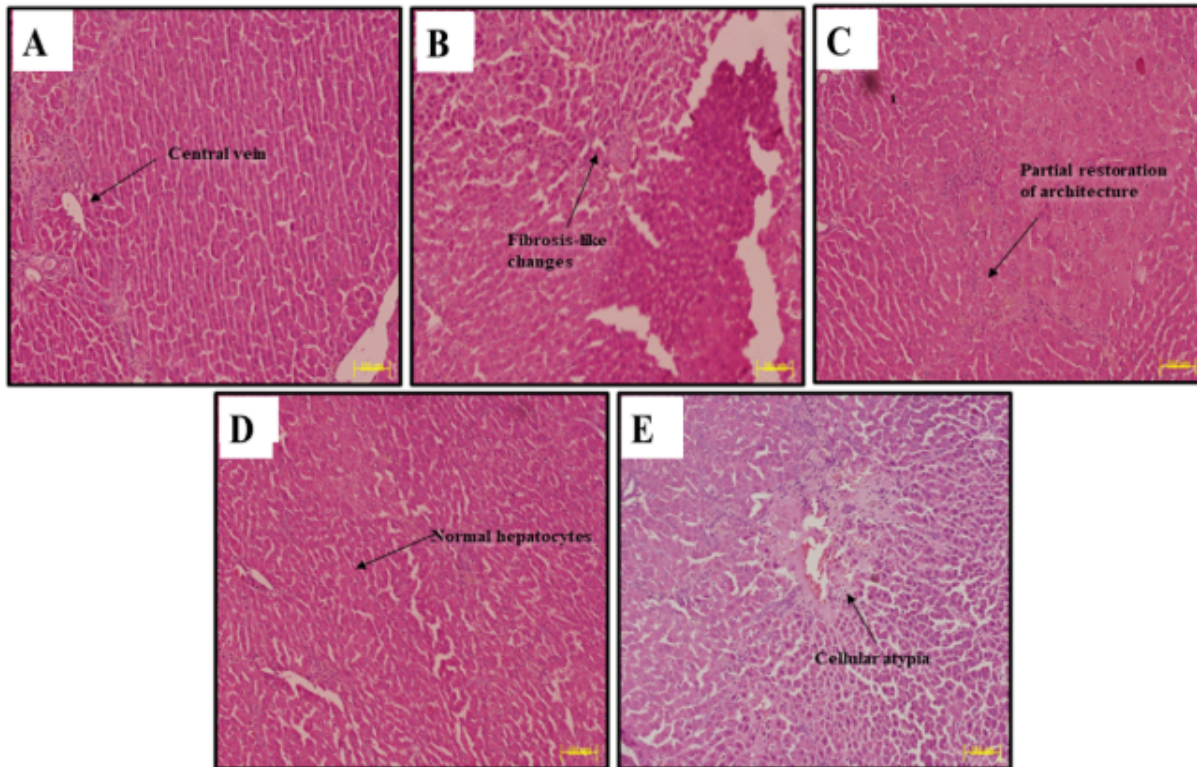


Figure 4

Histopathological analysis of liver tissues stained with hematoxylin and eosin (H&E). (A) Untreated control showing normal hepatic architecture with well-organized hepatic cords, sinusoids and normal hepatocytes. (B) Disease control showing hepatocellular necrosis, cellular atypia, disrupted hepatic architecture and early fibrotic changes. (C) Free MP1-treated group showing partial restoration of hepatic architecture with mild cellular degeneration and reduced atypia. (D) MP1-loaded exosome-treated group showing signs of restoration of hepatic architecture with minimal necrosis and reduced cellular atypia. (E) Unloaded PDELN-treated group showing minimal restoration with persistent structural disorganization.

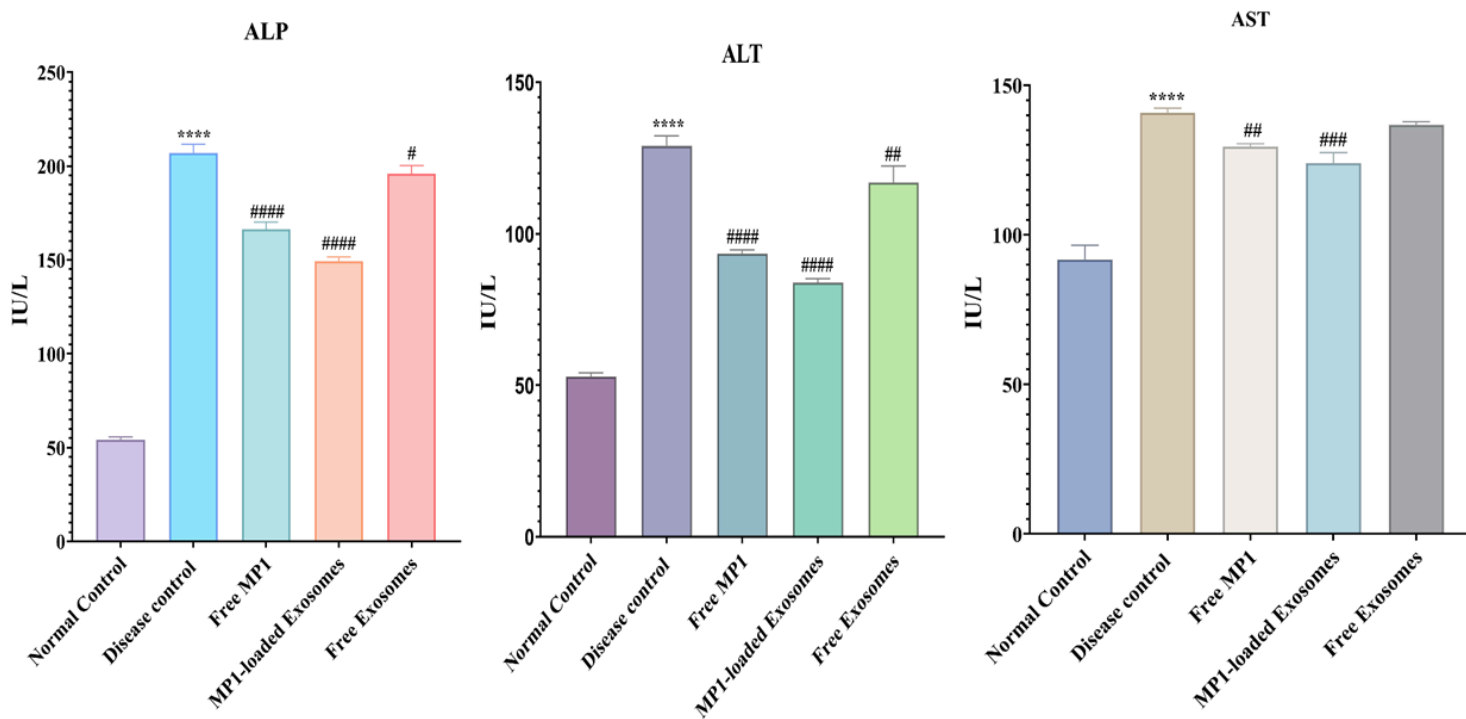


Figure 5

ALP, ALT, and AST levels in serum of Normal Control, Disease Control (DEN + CCl₄), Free MP1, MP1-loaded Exosomes and Free Exosomes groups. Data are presented as mean $\pm$ SD. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ indicate statistical significance between Normal Control and Disease Control groups. # $P \leq 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ represent comparison between Disease Control and treatment groups.