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Preparation and characterization of PBS (Polybutylene Succinate) nanoparticles containing cannabidiol (CBD) for anticancer application

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ABSTRACT

Cannabidiol (CBD), a major constituent of Cannabis sativa, has demonstrated a broad range of therapeutic properties in human studies. Notably, CBD has shown anticancer activity in preclinical cancer models. However, its low water solubility poses challenges for bioavailability, necessitating the development of drug delivery systems to enhance its efficacy. This study aimed to create CBD-loaded Poly (butylene succinate) (PBS) nanoparticles and evaluate their effectiveness in in vitro cancer models. The nanoparticles, with an average size of 175 nm, were produced using a modified double emulsion/solvent evaporation technique. The release profile of CBD from the nanoparticles exhibited an initial rapid release followed by a slower sustained release. Cytotoxicity assays demonstrated that the CBD-PBS nanoparticles retained the anticancer effects of free CBD, selectively reducing the viability of cancer cell lines without affecting non-transformed fibroblasts. Additionally, the nanoformulation modulated key cellular pathways, as indicated by decreased AKT phosphorylation and increased LC3-II levels, suggesting that the encapsulated CBD preserved its ability to induce autophagy-mediated cell death in cancer cells. The nanoformulation also effectively inhibited cell migration in highly invasive prostate cancer cells, mirroring the effects of free CBD, while not impacting the migration of non-tumoral fibroblasts. These results underscore the therapeutic potential of this CBD nanoformulation, setting the stage for further in vivo investigations.

1. Introduction

Cannabidiol (CBD), the primary non-psychoactive component of the *Cannabis Sativa* plant, has been highlighted as a potential therapeutic agent for various conditions, including cancer [1–6], epilepsy [7], and as a vasorelaxant, antipsychotic, antispasmodic, antiemetic, anti-ischemic, antibacterial, and anxiolytic agent [8]. The selective action of CBD against cancer cells, as opposed to non-tumoral fibroblasts, is thought to be due to the higher metabolic demands of cancer cells which may lead to a differential uptake or sensitivity to the compound [9–11]. Beyond its therapeutic effects, CBD has also been found to exhibit strong antioxidant properties, making it a potentially useful agent for reducing

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oxidative stress and inflammation [12]. Studies have shown that CBD is a more potent antioxidant than ascorbate and α-tocopherol [13]. However, the potential of CBD as a biomedical treatment is limited by its low aqueous solubility which can limit its effectiveness as a therapeutic agent. Furthermore, CBD is susceptible to presystemic metabolism, which can break it down before reaching its target site in the body. To overcome these challenges, researchers have sought innovative approaches such as nanotechnology-based methods to enhance the delivery of CBD to its target site [14].

The administration of CBD often requires the use of organic solvents or dispersing agents, which may increase its toxicity and limit the maximum dose that can be administered. To address this challenge, nanoparticles have emerged as a promising tool for the safe delivery of CBD in biomedical treatments. By incorporating CBD into nanoparticle carrier systems, we not only aim to enhance its solubility and provide protection from presystemic metabolism but also to preserve and fully leverage its intrinsic efficacy. This encapsulation strategy facilitates the administration of CBD without the need for concomitant organic solvents. Moreover, it is designed to improve the bioavailability of CBD in vivo through the enhanced permeability and retention (EPR) effect, ensuring more efficient drug delivery to tumor sites. In this way, the encapsulation does not artificially amplify the intrinsic activity of CBD but ensures its optimal delivery and efficacy in the target environment [15]. In this regard, while the use of nanoparticles has been shown to enhance CBD distribution primarily at the tissue level, leveraging the EPR effect for improved accumulation in tumor sites, specific cellular targeting within these tissues may require additional strategies beyond the EPR effect. These strategies could include surface modification of nanoparticles with ligands or antibodies that recognize and bind to molecules expressed on the target cells [15,16]. Thus, efforts have been made in recent years to optimize a nanoencapsulation approach for CBD which aim to exploit the EPR effect for better targeting of the drug, potentially leading to improved therapeutic outcomes in cancer treatments [14]. In this context the use of biodegradable and biocompatible polymers as a nanoparticle carrier system for CBD emerges as a safe and effective delivery system for this agent.

In the medical field, several polymers such as polylactic acid (PLA), poly(lactic-*co*-glycolic acid) (PLGA), and chitosan are commonly used as biomaterials due to their unique properties including biocompatibility, biodegradability, and versatility in design. However, the environmental impact of polymer usage cannot be ignored, and to address this issue, researchers have been developing polymerization processes based on monomers from renewable resources to produce biodegradable particles [17]. The use of biodegradable polymers in medicine not only reduces environmental impact but also offers a range of benefits including controlled drug release, targeted delivery, and biocompatibility [18]. Therefore, the development of sustainable polymer-based biomaterials is crucial for maintaining a healthy environment whilst advancing medical technology [19].

Accordingly, biomass has been highlighted as a source of a wide range of compounds, including succinate that may be used as chemical platforms for producing more complex polymerized structures. Poly (butylene succinate) (PBS) is an aliphatic polyester that can be obtained through the polycondensation of succinic acid and 1,4-butanediol [20]. PBS exhibits biodegradability and good thermal and mechanical properties. Moreover, the degradation products of PBS are not harmful to the environment or human health. Therefore, PBS is an excellent candidate for the development of nanocarriers that can be used for biomedical purposes. Moreover PBS production from succinic acid derived from biomass can contribute to reducing the environmental impact of conventional plastic production [20].

While PBS is a relatively recent material for drug delivery applications, its potential, particularly in the form of polymeric particles, is beginning to be explored. It is crucial to distinguish between the different scales of these particles. For instance, a research consortium has successfully encapsulated paliperidone palmitate in PBS to form microparticles, using the oil-in-water emulsification/solvent evaporation technique. These microparticles were within the size range of 20–50 μm, showcasing an entrapment efficiency of 60 % [21]. It is essential to consistently report these sizes as they significantly impact the distribution, efficacy, and safety profile of the drug delivery system. Further research is needed to bridge these gaps and fully elucidate the capabilities and characteristics of PBS in various forms and sizes, from micro to nanoparticles. Likewise, PBS was used to encapsulate levodopa for the treatment of Parkinson's disease, obtaining microspheres with a size of approximately 20 μm and a maximum encapsulation efficiency of 63 % [17]. In an alternative study, PBS microparticles were produced and functionalized with poly(hydroxypropyl methacrylate) (PHPMA) incorporating meloxicam via the emulsification and solvent evaporation technique, resulting in an encapsulation efficiency of 79 % [22]. Lastly, PBS-based copolymers nanoparticles were synthesized and successfully utilized to evaluate the potential of these polymers for controlled drug delivery systems by the encapsulation of Dexamethasone [23].

Although distinct studies discussed the advantages of using PBS as a matrix for drug encapsulation, including its biocompatibility, biodegradability, and tunable properties, there is still a lack of research exploring the potential of PBS for encapsulating cannabinoids. Given the increasing interest in the therapeutic use of cannabinoids, investigating PBS as a potential carrier for cannabidiol could be a promising avenue for future research in drug delivery. In this way, the main objective of the present study was to develop and assess the potential of PBS nanoparticles as a drug delivery system for CBD in biomedical applications.

2. Materials and methods

2.1. Materials

Polybutylene succinate (PBS, Mw = 4,580 g/mol) was prepared by suspension polycondensation process reaction [20,24]. CBD was purchased from THC-Pharm (Frankfurt, Germany, 99 % purity); polyvinyl alcohol (PVA, Mw = 30,000–70,000 g/mol), Sigmacote®, HPLC-grade acetonitrile, dichloromethane (DCM), dimethyl-sulfoxide (DMSO), chloroform and methanol were purchased from Fisher Scientific (Frederick, MA, USA); phosphate buffered saline solution (pH 7.4), 3-(4, 5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT); dulbecco's modified eagle medium (DMEM) were bought from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum was bought from Biowest (FBS, Biowest, Nuaillé, France); and, isopropyl alcohol was purchased from Fisher (Fisher Chemical, Pittsburgh, Pennsylvania, USA). All chemicals and reagents were used as received.

2.2. Preparation of PBS nanoparticles

PBS polymer was synthesized through suspension polycondensation of succinic acid and 1-4-butanediol. All development and characterization processes can be found in the work of Dutra et al. [20,25–27].

The development of poly(butylene succinate) (PBS) nanoparticles was achieved through an adaptation of the double emulsion/solvent evaporation method described by Brunner et al. [28]. Firstly, to prepare the organic phase, PBS polymer (100 mg) was solubilized in 1.8 mL of chloroform using magnetic stirring (300 rpm) at room temperature. Cannabidiol (CBD) (4 mg) was dissolved in the organic phase when used. Then, 6 mL of a 2 % (w/v) aqueous solution of polyvinyl alcohol (PVA) was added to the organic phase under magnetic stirring for 5 min. This emulsion was sonicated in an ultrasonicator (Fisher Scientific sonicator, Fisher Scientific, Frederick, MA, USA), at an amplitude of 60 %, 10 on and 20 off cycles for 4 min. The resulting emulsion was added to 40 mL of an aqueous 0.5 % (w/v) PVA solution with magnetic stirring for 3 h at 500 rpm and room temperature to completely evaporate the organic solvent. Following, to collect the nanoparticles, they were centrifuged for 30 min at 15,000 × rpm using a Beckman Coulter Avanti centrifuge (Beckman, California, CA, USA), and the supernatant was

removed. To provide cryoprotection, 1 mL of 3 % (w/v) sucrose was added, and finally, the samples were freeze-dried for 24 h at −50 °C and 0.2 mbar (Benchtop Freeze Dryer LyoQuest, Telstar). This freeze-drying process was critical for the preservation and subsequent characterization of the nanoparticles. Following centrifugation at 15,000×*g* for 30 min, the supernatant was removed. The addition of a 3 % (w/v) sucrose solution served as a cryoprotectant. The samples were then subjected to a freeze-drying process for 24 h at −50 °C and 0.2 mbar, facilitating the sublimation of ice directly from the solid to the gas phase under vacuum, followed by desorption to remove bound water molecules, thereby preserving the structural integrity of the nanoparticles for further analysis.

2.3. Characterization of PBS nanoparticles

2.3.1. Size distribution and zeta potential

The mean particle size was measured using a Microtrac®-Zetatrac™ Particle Analyzer (Microtrac Inc., Montgomeryville, PA, USA) by dynamic light scattering. The zeta potential was measured upon dilution in water using a Zetasizer Lab Blue Label (Malvern Panalytical) by electrophoretic light scattering. Zeta potential measurements were conducted on the lyophilized samples of the PBS nanoparticles. After reconstitution in an appropriate solvent, the lyophilized samples were carefully prepared to ensure accurate zeta potential analysis, reflecting the colloidal stability of the nanoparticles in their intended delivery medium.

2.3.2. TEM analysis

The morphological examination of PBS nanoparticles was performed by transmission electron microscopy (TEM). TEM images were taken on a JEOL JEM 1400 microscope. To prepare the samples for TEM, 20 μL of PBS nanoparticles suspension was dropped on a carbon copper grid (300 mesh) and dried at room temperature. The microscope was operated at an acceleration voltage of 120 kV.

2.3.3. Drug content, encapsulation efficiency, and loading capacity

CBD encapsulation analysis was performed using an HPLC system (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a reverse-phase Mediterranea® C18 (15 × 0.46 cm i.d., pore size 5 μm) (Teknokroma®) column. The mobile phase consisted of methanol:acetonitrile:water at pH 4.5 (52:30:18 v/v), and detection was carried out at a wavelength of 228 nm. A flow rate of 1.8 mL/min was used with an injection volume of 20 μL [29].

To quantify the amount of CBD encapsulated, 20 mg of lyophilized nanoparticles were dissolved in a mixture of dichloromethane (DCM) and methanol by vortexing; afterwards, the resulting solution was filtered through a 0.45 μm filter (Polypropylene, Filter-Lab®) and analyzed by High-Performance Liquid Chromatography (HPLC).

The encapsulation efficiency (EE) and drug loading capacity were calculated using the following equations (I) and (II):

$$EE(\%) = \frac{(CBD : PBS\ experimental\ ratio)}{(CBD : PBS\ theoretical\ ratio)}.100 \quad \text{(I)}$$

$$Drug\ Loaded\ mgCBD \Big/ 10mgNp = \frac{10(CBD : PBS\ experimental\ ratio)}{total\ weight\ of\ teorical\ formulation} \quad \text{(II)}$$

The 'CBD:PBS experimental ratio' was calculated by combining the weight of the total amount of PBS nanoparticles obtained after the lyophilization process with the measured CBD content from HPLC analysis. By comparing this ratio with the 'CBD:PBS theoretical ratio' (the ratio of CBD to PBS initially used in the formulation), the EE can be accurately determined.

To determine the optimal ratio of CBD to PBS for encapsulation, we prioritized achieving a concentration of CBD that could be therapeutically relevant. Although higher CBD loadings could have been potentially explored, the current study was not aimed at experimentally determining the maximum possible load of CBD.

2.3.4. In vitro drug release studies

To study the drug release from nanoparticles, 20 mg of freeze-dried nanoparticle was solubilized in 2 mL of phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.5 % (w/v) Tween® 80. The concentration of CBD used in this study did not exceed the solubility limit of CBD in the release medium, thus maintaining sink conditions throughout the study. CBD solubility in PBS with Tween® 80 is referenced in the scientific literature, which has been cited in the manuscript [30]. This concentration ensured that the amount of CBD corresponding to the 20 mg of nanoparticles in 2 mL of release medium was sufficient for achieving sink conditions. The nanoparticle suspension of 10 mg/mL was then maintained in a thermostatic bath at 37 ± 0.5 °C, agitated at 100 rpm over 72 h, under sink conditions [30].

To address concerns regarding the potential sampling of nanoparticles during the release study, we implemented a rigorous centrifugation protocol. Samples were withdrawn at pre-defined time points (0.25, 1, 2, 3, 6, 24, 48, and 72 h), centrifuged at 10,000 × rpm (Beckman Coulter Avanti centrifuge, Beckman, California, CA, USA) for 15 min. This centrifugation step was sufficient to sediment the nanoparticles and ensure that the supernatant, which was subsequently filtered through a 0.45 μm filter (Polypropylene, Filter-Lab®), contained only the released CBD. The filtered supernatant was then analyzed by HPLC [16]. Calibration curves of standard CBD in different concentrations were used to realize the HPLC analysis. The release data were analyzed using mathematical models such as zero-order (Qt = Q0+K0t), first-order (lnQt = lnQ0+K1t), Korsmeyer–Peppas (M1/M∞ = Ktn), and Higuchi (Qt = KHt1/2) [31]. We ensured the accuracy of the drug release profile by employing these stringent methods, thereby mitigating any misinterpretation of the results that could arise from nanoparticle depletion. Excel software was used to analyze the linear regression in terms of intercepts, slopes and coefficients of determination (R^2). The model with the best fit was determined by the highest value of the coefficient of determination.

2.4. In vitro biological studies

2.4.1. Cell lines

The following human cell lines B16–F10, U118MG, U87MG, DU145, PC3, were purchased from the American Type Culture Collection. Normal (Non tumor associated) fibroblasts (NF) were kindly donated by Akira Orimo. These cells were obtained from a woman undergoing reduction mammoplasty [32]. All cell lines were cultured in DMEM (NF, U87MG, U118MG) or RPMI (B16–F10, DU145, PC3) and supplemented with 10 % wt. fetal bovine serum (FBS) and 1 % penicillin/streptomycin and maintained under standard culture conditions (37 °C in a humidified atmosphere with 5 % CO_2).

2.4.2. Viability assay

To carry out the different experimental procedures the cells were initially seeded at a density of 5 × 10^3 cells/well in 96-well plates in medium containing 10 % FBS. After 8 h the medium was removed, substituted by medium containing 0 % FBS serum and cell were incubated under standard culture conditions (37 °C, 5 % CO_2, and 95 % humidity) for 24 h to ensure that all serum had been removed from the medium. Subsequently, cells were subjected to the different treatments [free CBD, PBS, and CBD-loaded PBS nanoparticles] and incubated for another 24 h. After the treatment period, the culture medium was removed, and the number of viable cells was estimated using the MTT test. This colorimetric assay quantitatively measures the mitochondrial metabolic activity that enzymatically reduces the soluble MTT to an insoluble formazan product. Specifically, cells were incubated with 100 μL of an MTT solution (0.5 mg/mL), a concentration optimized for our specific cell lines, for 3 h to facilitate the formation of formazan crystals.

Following this, 100 μL of isopropyl alcohol was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using a SpectraMax® M3 microplate reader. To ensure reproducibility, all experiments were performed in triplicate and the results were expressed as the percentage of viable cells compared to the control group, which were cells treated with 0.2 % v/v DMSO (established as non-toxic in preliminary experiments), serving as the 100 % viability reference.

2.4.3. Western blot

Western blot analysis was performed using standard procedures. Briefly, proteins were extracted using Radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1 % (v/v) NP40, 50 mM Tris-HCl pH 8.0, 0.1 % (v/v) SDS, 1 mM EDTA, 0.5 % (w/v) deoxycholate]. Total protein concentration was determined using the Bradford method. Proteins were separated by SDS-PAGE on 12 % acrylamide gels (Bio-Rad, Hercules, CA, USA), transferred to polyvinylidene difluoride membranes, and blocked with either a 5 % skim milk solution or 5 % BSA (Sigma) at 4 °C overnight with primary antibodies. The membranes were then probed with the following primary antibodies: anti-pAKT S473 (1:1000; Cell signaling, #9271, Danvers, MA, USA), anti-AKT (1:1000; Cell signaling, #9272, Danvers, MA, USA), anti-LC-3 (1.2000; Sigma-Aldrich; #L7543), anti-actin (1:4000; Sigma; A5441, St. Louis, MO, USA). Antibody binding was detected with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000; GE Healthcare, Chicago, IL, USA) and visualized by enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA). The images were captured with the ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, Chicago, IL, USA). The enhanced chemiluminescence (ECL) results were scanned, and the amount of each protein band was quantified using NIH Image J software (NIH Image, Bethesda, MD, USA, http://rsb.info.nih.gov/nih-image/).

2.4.4. Wound healing assay

3×10^5 DU145, PC3 and NF cells per well were seeded in a 6-well culture plate for 48 h, to create a uniform cell monolayer. After that, a linear scar was generated on the cellular layer by using a pipette tip, the medium was absorbed with vacuum, and the wells were cleaned twice with PBS without Ca^{2+} or Mg^{2+}. Finally, the cells were incubated with the following treatments: vehicle (CT), free CBD, PBS-NPs and PBS-encapsulated CBD resuspended in cell culture medium at the concentrations reported. The gap migration was imaged at 0 (t_0) and 24 h (t_1) using Leica DM4 B Fluorescence LED Microscope, and the images were quantified with ImageJ using "wound healing size tool" plugin. The scratched area values at t_1 were normalized to corresponding images at t_0.

2.5. Statistical analysis

Statistical significance was assessed using one-way ANOVA analysis, (Tukey's test) or unpaired *t*-test two tailed ($p < 0.05$) using Prism software version 8.0, GraphPad. All experiments were carried out at least in triplicate ($n = 3$) and the results were calculated as mean ± standard error mean (SEM) or ± standard deviation (SD) as reported in the figure legend in each case.

3. Results

3.1. Preparation of PBS nanoparticles and drug encapsulation

The PBS nanoparticles containing CBD were successfully produced using an adapted double emulsion/solvent evaporation technique [28]. As shown in Table 1 and Fig. 1 control (empty) and CBD-loaded nanoparticles exhibited a similar mean diameter of approximately 175 nm, which lies within acceptable limits for biomedical applications [33].The analysis indicates a polydispersity distribution characterized by the presence of two peaks in the size distribution profile, with the primary peak representing 90 % of the population. Polydispersity in nanoparticle analysis refers to the degree of non-uniformity in the size distribution of a nanoparticle sample and measures the distribution of individual particle sizes within that sample. Importantly, the analysis suggests that the observed polydispersity may be attributed to the formation of aggregates rather than the fusion of nanoparticles, as evidenced by the TEM images (Fig. 1, C and F). It is worth mentioning that despite a negative zeta potential of −25 mV, which theoretically precludes coalescence from occurring, approximately 10 % of the nanoparticles were found as aggregates. In addition, CBD showed an encapsulation efficiency of (>90 %) (Table 1). These values can be attributed to the high hydrophobicity of CBD, which is incorporated more easily in hydrophobic polymer solutions [2,28], and indicate that the method selected for CBD encapsulation was appropriate.

Table 1
Volume diameter of nanoparticles (Dp) obtained by DLS; zeta potential (ZP) obtained by ELP, encapsulation efficiency (EE) and Drug Loading in each 10 mg of Np.

Sample	Dp (nm)	ZP (mV)	EE (%)	Drug Loading mgCBD/ 10mgNp
PBS-NPs	177.7	−23.34 ± 4.10	–	
PBS-NPs-CBD	175.4	−30.35 ± 4.30	90.1 ± 12.0	0.35 ± 0.05

3.2. Drug release analysis

The release profile of the PBS nanoparticles containing CBD was determined *in vitro* over 72 h. To confirm that sink conditions were achieved, the solubility of CBD in the release medium was established, and the volume of release medium used was sufficient to maintain these conditions. As illustrated in Fig. 2, the release profile displayed an initial burst release phase, with approximately ≈50 % of the CBD released in the first few hours, followed by a slow-release rate phase. This initial burst release is a common characteristic of nanoparticles and can be attributed to the rapid release of the drug molecules located on the surface or close to the surface of the nanoparticles [34]. Fraguas-Sánchez et al. (2020) observed a similar behavior in PLGA nanoparticles containing CBD, where the nanoparticles exhibited a high burst effect in the first hour, with approximately 35 % of the CBD released.

The absence of nanoparticles in the sampled solution was ensured by the centrifugation and filtration process before HPLC analysis. The release of the encapsulated drug from nanoparticles is influenced by multiple factors, including particle geometry, size, and the nature of the encapsulating agent. The size of the particles is particularly crucial as it can significantly impact the surface area to volume ratio, thereby affecting the rate and pattern of drug release. Together, these factors dictate the release mechanism, which can occur through various processes such as swelling, erosion, diffusion, degradation, and solvent effects. The interplay of these elements determines the suitability of the drug delivery system for specific therapeutic applications [31,35]. Our methodology, including the decision not to use dialysis, was chosen to provide an accurate representation of the release kinetics in a system that closely mimics physiological conditions.

3.3. Mathematical analysis of kinetic assay

The kinetics of the drug release from polymeric nanoparticles can be better understood by mathematical model's analysis. In this study, several models, including the zero-order, first-order, Korsmeyer–Peppas, and Higuchi models were utilized.

The release kinetics of CBD from PBS nanoparticles can be accurately described by the Korsmeyer–Peppas equation followed by the Higuchi

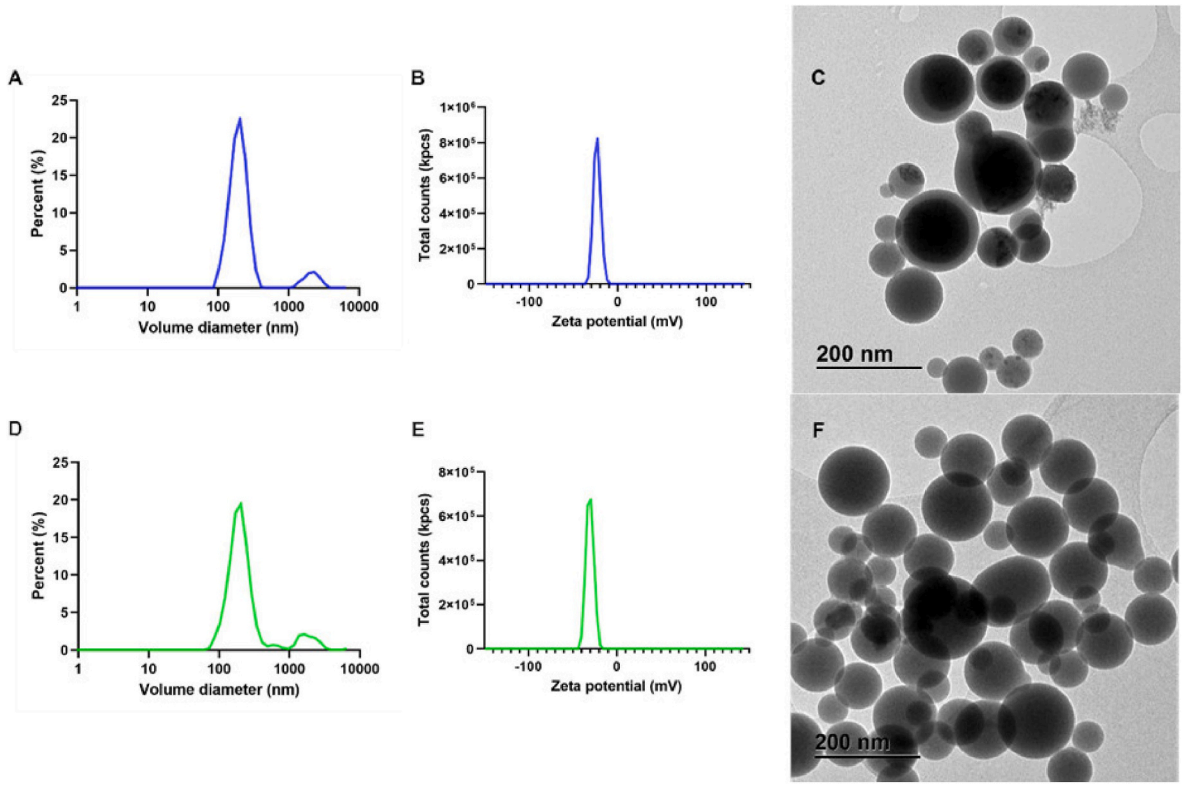

Fig. 1. Characterization of PBS nanoparticles (blank: A-C; and CBD-loaded: D-F): volume diameter distribution (A, D); zeta potential distribution (B, E) and TEM images (C, F).

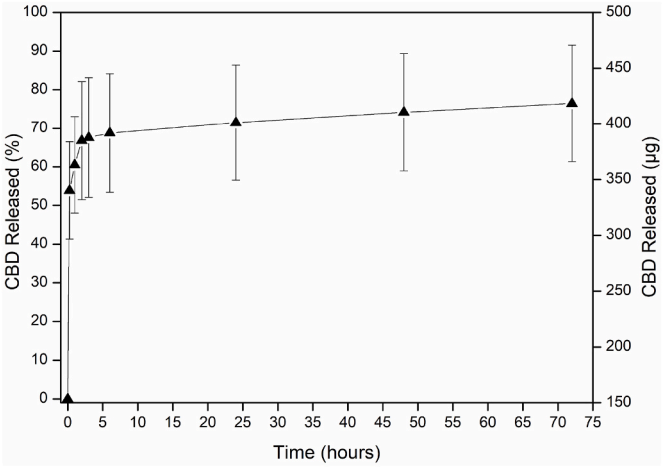

Fig. 2. The release profile of CBD-PBS nanoparticles obtained in phosphate buffered saline pH 7.4 (0.5 % Tween 80). Data refer to the mean ± standard deviation (n = 3).

Table 2
Mathematical Models and Kinetic Parameters. The table presents the correlation coefficient (R2), the rate constant (k), and the release exponent (n) for different mathematical models applied to describe the drug release kinetics.

Mathematical models			
	R^2	k	n
Zero order	0.621	16.996	–
First order	0.564	0.004	–
Higuchi	0.734	0.349	–
Korsmeyer–Peppas	**0.928**	8.357	**0.143**

model (Table 2). The diffusional exponent value below 0.5 suggests that the release of CBD from PBS nanoparticles occurs via a diffusion mechanism through the polymer matrix. These results support the fact that, among all models tested, the Higuchi model, based on Fickian's diffusion, is the one with the highest coefficient of determination. Overall, PBS nanoparticles represent a promise for delivering CBD in biomedical applications.

3.4. Evaluation of in vitro cytotoxicity in cancerous and non-cancerous cell lines

In our initial evaluation of the anticancer potential of the CBD-PBS nanoparticles, we focused on their impact on cell viability across various cell lines, including one melanoma (B16–F10) and two glioma lines (U118MG, U87MG). Our data, shown in Fig. 3 and Figure S5, reveal that CBD-PBS treatment, in contrast to the control (empty-PBS) nanoparticles, resulted in a dose-dependent decrement in viability for all cancer cell lines tested. Notably, the empty-PBS nanoparticles induced a slight, yet measurable, decrease in viability in B16–F10 melanoma cells, albeit to a lesser extent compared to the CBD-loaded nanoparticles.

In contrast, the viability of normal fibroblasts remained unaltered upon treatment with the CBD-loaded nanoformulation, as shown in Figure S5. This latter observation supports the idea that cytotoxicity elicited by CBD is selective towards cancer cells over non-tumoral cells. Collectively, these results suggest that the PBS nanoformulation can deliver CBD effectively, while concurrently maintaining its intrinsic selectivity towards cancer cells. Additionally, as illustrated in Figure S6, the chemically synthesized PBS polymer itself did not exhibit cytotoxicity in either cancerous or non-cancerous cell lines, reinforcing the notion that PBS is a biocompatible and efficacious nanocarrier.

3.5. Analysis of cell proliferation and autophagy markers

The AKT/MTORC1 signaling pathway plays a critical role in

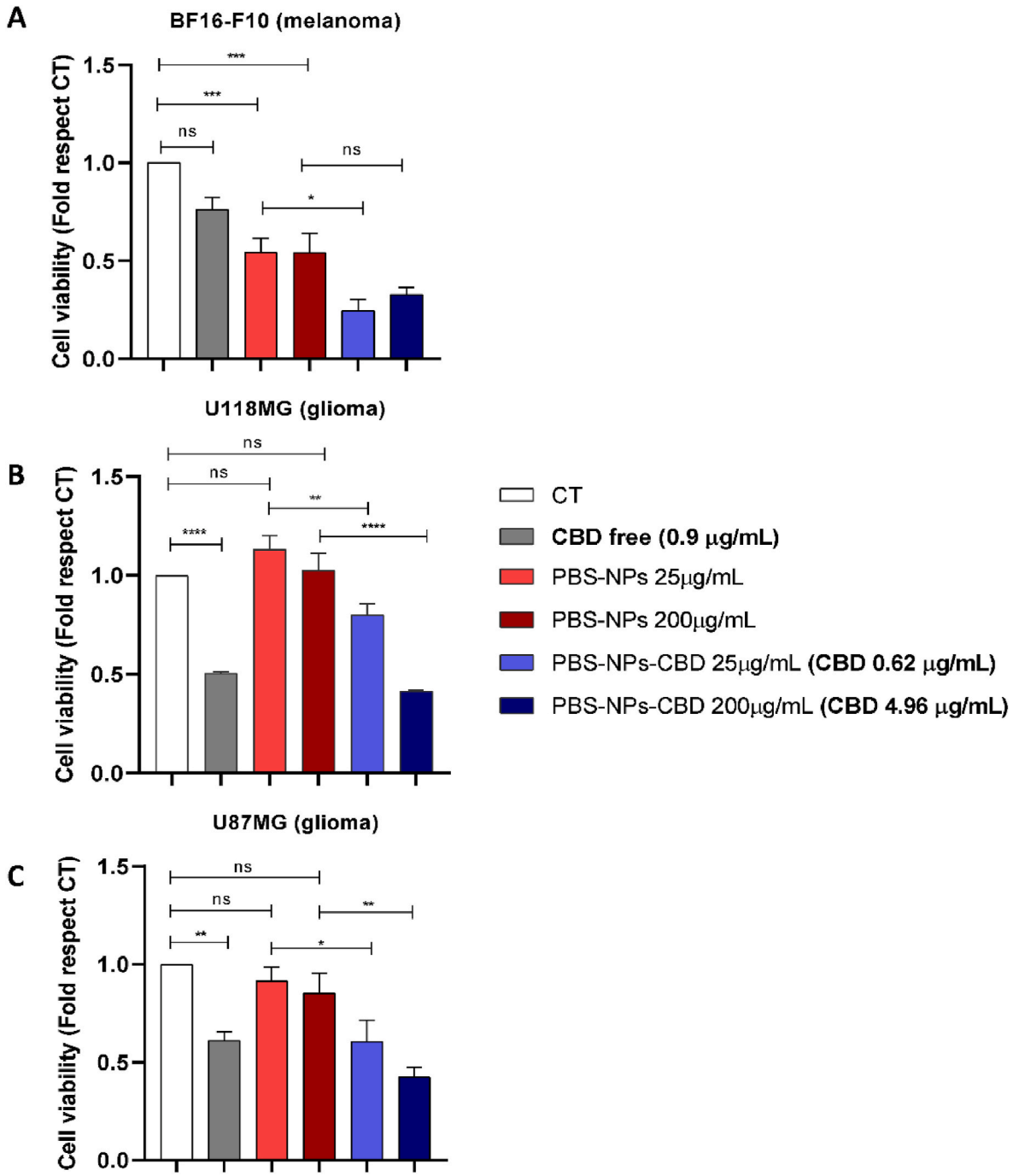

Fig. 3. Cytotoxicity assays in cancer cell lines. (A–C) Effect of treatment for 24 h with free (no encapsulated) CBD (0.9 μg/mL), empty nanoparticles (PBS-NPs 5 μg/mL and 200 μg/mL) and nanoparticles carrying CBD (PBS-NPs-CBD 5 μg/mL and 200 μg/mL, which corresponds to a CBD concentration of 0.62 μg/mL and 4.96 μg/mL, respectively) on the viability (as estimated by using the MTT test) of BF16–F10 (upper panel), U118MG (middle panel) and U87MG (bottom panel) cells. The values are reported as fold change ± standard error (S.E.) of at least three independent experiments. Significant differences are shown *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$ (unpaired one-way ANOVA followed by Tukey test).

regulating cell proliferation and growth, and its dysregulation is known to contribute to tumorigenesis [36–38]. Therefore, targeting this pathway represents a promising anticancer therapy [39]. One of the cellular mechanisms by which this signaling axis regulates these processes is the control of autophagy, a cellular process by which different cellular components are targeted for degradation to the lysosomes. The AKT/MTORC1 axis is a potent inhibitor of autophagy via multiple mechanisms [40,41]. Of importance, under certain cellular contexts the inhibition of the AKT/MTORC1 axis and the subsequent stimulation of autophagy can lead to cancer cell death [42,43]. Specifically, previous work has shown that CBD can promote autophagy-mediated cancer cell death by inhibiting the AKT/MTORC1 axis [9,44–47]. Therefore, we next investigated whether the encapsulated CBD affected the survival of cancer cells by altering this signaling mechanism. To this aim, we assessed AKT phosphorylation as well as the lipidation of LC3 (the lipidated – phosphatidylethanolamine-conjugated - and autophagosome-associated form of LC3, named LC3-II, is a well-established readout of autophagy [40,48]). We observed that treatment with both free and PBS-encapsulated CBD induced a similar reduction of AKT phosphorylation in Ser 473, which was paralleled by

the accumulation of LC3-II in all three cancer cell lines tested (Fig. 4 and S7). While both free and encapsulated CBD effectively reduced AKT phosphorylation, these findings highlight that the nanoformulation maintains the intrinsic modulatory role of CBD on this pathway. Furthermore, the encapsulation may offer additional benefits, such as potentially prolonging the duration of action, although this aspect warrants further investigation to be conclusively determined. In contrast empty PBS nanoparticles did not affect LC3-II accumulation and only marginally impacted AKT phosphorylation, implying that these structures are relatively inert and serve as efficient nanocarriers for CBD delivery to target cells while minimizing toxicity.

3.6. Analysis of cell migration of prostate cancer cells

Different studies had previously reported that CBD exerts an inhibitory effect on cancer cell migration [9,49,50], an event that can serve as an indirect indicator of the potential metastatic capacity of cancer cells [51]. Consequently, to complete the characterization of the antitumoral activity of the PBS-CBD nanoparticles, we next investigated the ability of this encapsulated form of CBD to inhibit the migration of two highly invasive prostate cancer cell lines (DU145 and PC3) as well as of primary fibroblasts as a control of non-cancerous cells using the wound healing assay.

To carry out these analyses, we selected two concentrations (25 and

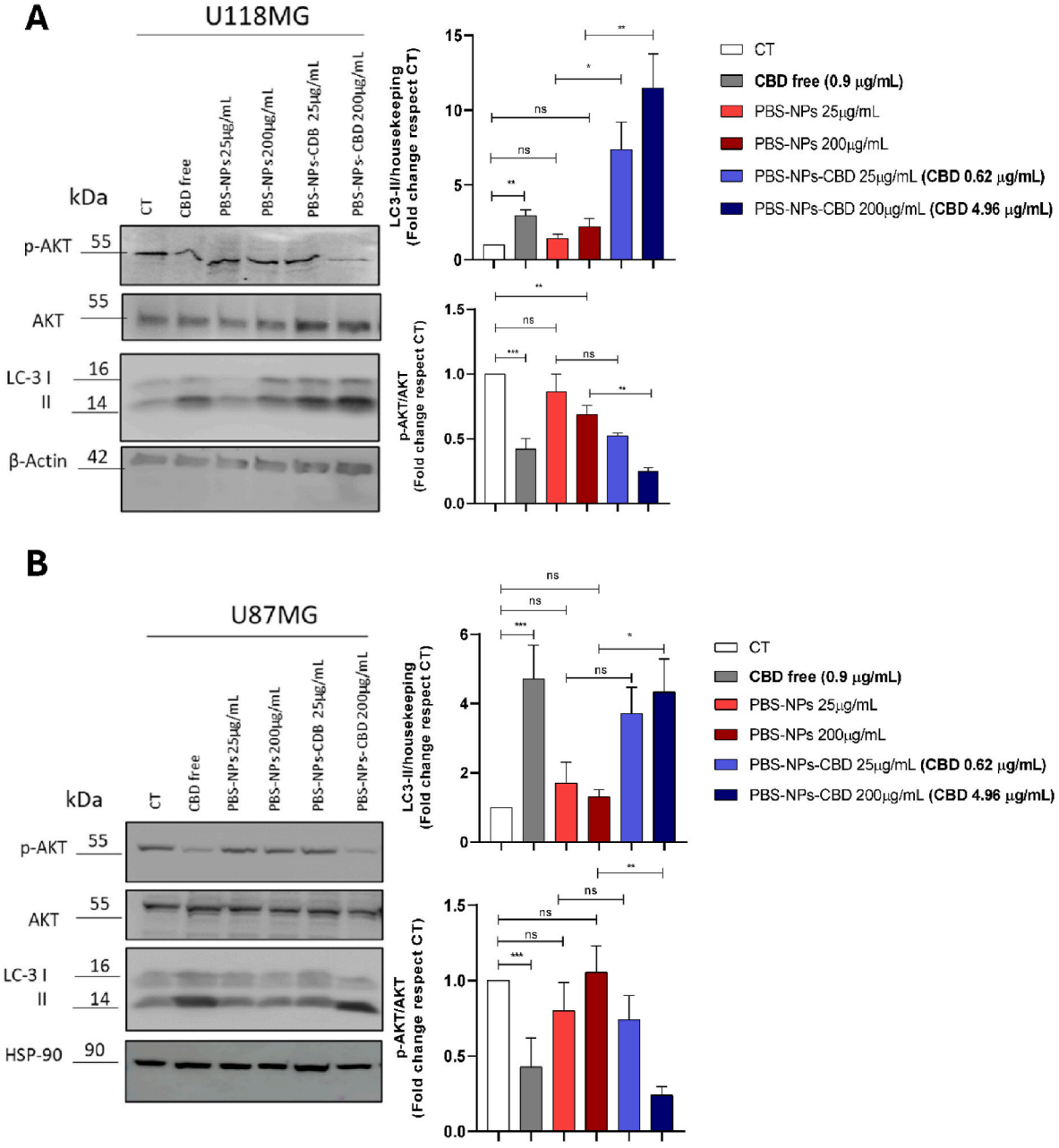

Fig. 4. Analysis of autophagy and proliferation markers. (A–B) Effect of treatment for 24 h with free (no encapsulated) CBD (0.9 μg/mL), empty nanoparticles (PBS-NPs 5 μg/mL and 200 μg/mL) and nanoparticles carrying CBD (PBS-NPs-CBD 5 μg/mL and 200 μg/mL, which corresponds to a CBD concentration of 0.62 μg/mL and 4.96 μg/mL, respectively) on the cell proliferation and autophagy markers of U118MG (upper panel) and U87MG (bottom panel) cells. Whole-cell extracts were processed for Western blot analysis of the indicated antibodies. β-Actin or HSP-90 protein levels were used as a control loading. Western bands were quantified with ImageJ as described in materials and methods and the values of quantification were reported as fold change ± standard error (S.E.) of at least three independent experiments. Significant differences are shown *p < 0.05; **p < 0.005; ***p < 0.0005 (unpaired *t*-test two tailed).

50 μg/mL) of CBD-PBS, that did not affect the viability of these cell lines. We found that the encapsulated form of CBD impaired the migration of DU145 and PC3 cells at a similar extent than non-encapsulated CBD (Fig. 5 and Figure S8). The results underscore the promise of the developed CBD nanoformulation, which is designed to improve bioavailability and targeting via the EPR effect. Importantly, it preserves the inherent efficacy of CBD in inhibiting cancer cell migration, ensuring that the therapeutic potential of CBD is fully harnessed and possibly enhanced in terms of delivery and bioavailability, compared to its free form. In contrast, when empty PBS nanoparticles were administered, no significant changes with respect to vehicle (CT) were observed. Notably, the effect of PBS-CBD on fibroblast migration was negligible (Figure S9). These data indicate that the developed CBD nanoformulation inhibits the migratory capacity of cancer cells but not of their non-transformed cell counterpart.

4. Discussion

Cannabidiol (CBD) has gained significant attention in recent years for its potential as an anticancer agent [52]. Its diverse range of therapeutic effects includes antiproliferative and pro-apoptotic actions, which can be beneficial in the management of various types of cancer [53]. However, the efficacy of CBD as a therapeutic agent in vivo is often hindered by its low solubility, rapid metabolism, and limited bioavailability [54]. This highlights the critical need for an effective delivery system to harness the full anticancer potential of CBD. Among the studies that encapsulated CBD in nanoparticles, none of them employed polybutylene succinate (PBS) as a matrix for encapsulation so far.

The current study presents a delivery system that employs PBS-based nanoparticles for CBD encapsulation, addressing the aforementioned limitations. The characteristic small size and large surface area of these nanoparticles may be a way of enhancing CBD stability, enabling targeted delivery and thus, maximizing its therapeutic potential. Nonetheless, in this study we have not carried out a complete characterization of CBD stability in the PBS nanoparticles at the long-term an issue that will have to be investigated in future studies in preclinical models of cancer. While our data suggest that the PBS nanoformulation enhances the delivery and bioavailability of CBD, particularly in potential future in vivo applications, it is crucial to clarify that the observed selectivity and anticancer effects are inherent characteristics of CBD itself. The nanoformulation primarily serves as a more efficient vehicle for the delivery of CBD, leveraging the EPR effect in tumor tissues, without inherently amplifying the anticancer properties of CBD.

Notably, the developed nanoformulation is potentially biodegradable [25], posing as an environmentally friendly solution that mitigates potential concerns regarding bioaccumulation and toxicity. This attribute not only underscores its environmental sustainability but also offers an advantage in pharmaceutical and clinical translation. In this regard, regulatory bodies such as the FDA often favor drugs and delivery systems with a favorable safety profile and minimal environmental impact [55]. Therefore, the developed biodegradable nanoformulation aligns with such preferences, potentially facilitating its approval and commercialization, and thereby expediting the clinical translation of CBD as an effective anticancer agent.

Like many pharmacological agents, CBD exerts its effects possibly through a complex interplay of interactions with various molecular entities within the cell, although the exact receptors and pathways are yet to be clearly delineated. Previous research has linked the cytotoxic effect of CBD to its ability to inhibit the AKT/MTORC1 axis and induce autophagy-mediated cancer cell death. Consistent with this notion, the present study demonstrates that PBS-encapsulated CBD reproduces the effect of non-encapsulated CBD and exerts cytotoxic effects in a panel of cancer cells. Furthermore, the developed nanoformulation inhibits AKT phosphorylation and induces LC3-II accumulation, which are established hallmarks of cell proliferation and autophagy activation, respectively. The presented data also reveals that encapsulated CBD reduces cell migration in highly invasive prostate cancer cells, thereby limiting their metastatic potential. It is worth noting that CBD elicits different

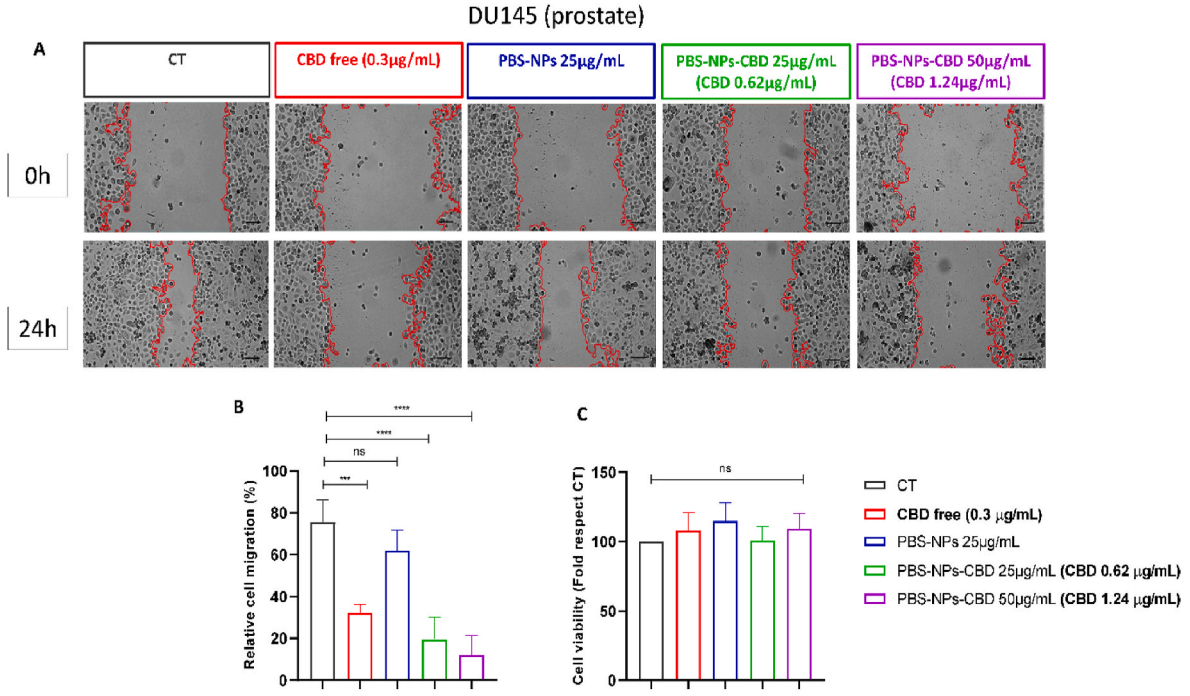

Fig. 5. Analysis of Cell migration. (A–C) Effect of treatment for 24 h with free (no encapsulated) CBD (0.3 μg/mL), empty nanoparticles (PBS-NPs 25 μg/mL) and nanoparticles carrying CBD (PBS-NPs-CBD 25 μg/mL and 50 μg/mL, which corresponds to a CBD concentration of 0.62 μg/mL and 1.24 μg/mL, respectively) on the cell migration (as estimated by using the wound healing assay) and cell viability (as estimated by using the MTT test) of DU145 cells. The images were quantified with ImageJ as described in materials and methods and reported as fold change ± standard error (S.E.) of at least three independent experiments. Significant differences are shown ***: $p < 0.001$; ****: $p < 0.0001$ (unpaired One-way ANOVA followed by Tukey test).

responses in cancerous and non-cancerous cells [11,56,57]. Whilst CBD treatment induces cancer cell death, it has been shown to exert a protective effect in various primary cultures of non-tumoral cells [58–60]. The specificity of CBD in affecting cancer cells over non-cancerous cells like fibroblasts can be attributed to the distinct metabolic pathways active in cancer cells, as well as to a different expression of cannabinoids receptors, which may result in differential uptake or sensitivity to CBD [3,61,62].

Whilst the current study presents evidence for the anticancer potential of CBD when delivered via PBS nanoparticles, several avenues of research remain to be explored in the future. Primarily, while the developed CBD nanoformulation showed promising anticancer effects *in vitro*, in vivo studies are critically needed to validate these findings in a more physiologically relevant environment. This would include the assessment of the therapeutic efficacy of the nanoformulations in animal models of glioma, melanoma, and prostate cancer. Another key area of future investigation is the long-term safety and potential toxicity of the developed nanoformulation. Whilst the present study demonstrated limited or negligible acute cytotoxic effects on non-tumoral cells, the long-term effects, remain unknown. Finally, the present study focused on AKT signaling pathways affected by the developed CBD nanoformulation. Since the effect of CBD and its nanoformulation could be more complex and involve other molecular pathways, future investigations should seek to elucidate other molecular pathways and targets potentially influenced by the developed nanoformulation, thereby providing a more comprehensive understanding of its mechanism of action.

5. Conclusion

A double emulsion/solvent evaporation technique was successfully adapted to develop PBS environmentally-friendly nanoparticles carrying CBD. This nano formulation permitted a 50 % release of the drug within the first few hours demonstrating its potential in controlled drug delivery. Importantly, while the PBS nanoformulation enhanced the delivery and bioavailability of CBD, the observed anticancer effects, such as the reduction in viability and migratory capacity of cancer cell lines and the inhibition of the AKT pathway, are primarily attributed to the inherent properties of CBD itself. These effects were notably absent in non-transformed cells, highlighting the selective action of CBD against cancer cells over non-cancerous cells. This specificity can be attributed to the distinct metabolic pathways in cancer cells, which may result in differential uptake or sensitivity to CBD. Overall, our findings support the idea that encapsulating CBD in PBS nanoparticles could be an interesting strategy to develop CBD-based medicines for anticancer purposes, particularly by improving its delivery to target sites.

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CRediT authorship contribution statement

Natália Freitas Freire: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Marco Cordani:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Conceptualization. **Juan Aparicio-Blanco:** Writing – review & editing, Methodology, Investigation. **Ana Isabel Fraguas Sanchez:** Writing – review & editing, Methodology, Investigation. **Luciana Dutra:** Writing – review & editing, Methodology, Investigation. **Martina C.C. Pinto:** Writing – review & editing, Methodology, Investigation. **Ali Zarrabi:** Writing – review & editing, Visualization, Methodology, Investigation. **José Carlos Pinto:** Writing – review & editing, Supervision, Conceptualization. **Guillermo Velasco:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Rosana Fialho:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2024.105833.

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