BIOPOLYMER NANOPARTICLES LOADED WITH CURCUMIN FOR BIOMEDICAL APPLICATIONS

Dana-Maria MIU[1,2], Fawzia SHA'AT[1], Georgeta NEAGU[1], Adrian ALBULESCU[1,3], Ramona-Daniela PAVALOIU[1], Mihaela-Carmen EREMIA[1], Sorin-Ion JINGA[2]

The study aimed to develop and assess curcumin-loaded polymeric nanoparticles using pullulan for biomedical applications. These nanoparticles were characterized for size, entrapment efficiency, polydispersity index, release kinetics, and stability. Cytotoxicity was assessed using a tetrazolium-based assay on various cell lines. The pullulan-based nanoparticles demonstrated nanometric size, narrow distribution, high entrapment efficiency (>80%), and excellent stability at 4 °C for over three months. Curcumin release was higher in neutral compared to slightly acidic medium, peaking at 72 hours. In vitro studies confirmed no cytotoxicity on tested cell lines, showcasing potential biomedical applications including cancer.

Keywords: pullulan; curcumin; biopolymeric nanoparticles; biomedical applications

1. Introduction

Researchers have recently become interested in biopolymeric nanoparticles (NPs) due to their unique features caused by their small size. [1-3].

The advantages of applying nanoparticles as drug carriers include the capacity to protect the drug and other physiologically active molecules from the environment, improve bioavailability, and therapeutic index [1,4]. NPs have several important advantages, including the capacity to: i) produce controlled release at the appropriate area; ii) increase the stability of labile molecules and iii) modify the surface with ligands for protection and accurate drug delivery.

A wide range of polymers can be used to create NPs for biomedical applications. While there are no precise protocols for establishing a polymer's capacity for medical application, the major criteria revolve around ensuring the polymer is non-toxic, biocompatible, and biodegradable [4,5].

Pullulan, a natural biopolymer derived from the microbial strain *Aureobasidium pullulans* by fermentation, was recognised as a substance with the ability to create drug nanocarriers. Because of its beneficial qualities, including

[1] National Institute For Chemical-Pharmaceutical Research&Development – ICCF, Bucharest, Romania; e-mail: fawzya.shaat@gmail.com; getabios@yahoo.com; dana.miu92@gmail.com; pavaloiu_daniella@yahoo.com;

[2] Faculty of Chemical Engineering and Biotechnologies, National University of Science and Technology POLITEHNICA Bucharest, Romania; e-mail: dana.miu92@gmail.com; sorinionjinga@yahoo.com;

[3] Department of Molecular Virology, Stefan S. Nicolau Institute of Virology, Bucharest, Romania; e-mail: adrian.albulescu@virology.ro;

biodegradability, biocompatibility, non-mutagenicity, non-immunogenicity and non-carcinogenicity, pullulan is appropriate for a range of biomedical applications [6,7]. Pullulan may go through functionalization or derivatization by several chemical reactions such etherification, amidification, sulfation, esterification, oxidation, and copolymerization to substitute the hydroxyl groups with desired moieties [9,10]. Hydrophobized pullulan is commonly utilised as a drug delivery carrier due to its inability to self-associate in aqueous solutions caused by its solubility in water. Pullulan-based nanoparticles function as nanocarriers for an extensive range of therapeutic substances, including essential oils and antimicrobial agents that exhibit inhibitory activity against Gram-negative and Gram-positive pathogenic fungi and bacteria, including those that are biofilm-forming and multidrug-resistant [8]. For anticancer agents to target organs including the liver, spleen, lungs, brain, and more, pullulan and its derivatives serve as excellent carriers. They also facilitate the continuous or extended release of particular cytotoxic chemicals at the disease site [11].

Curcumin, scientifically named 1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione, [12] is a polyphenol obtained from the rhizome of turmeric root (*Curcuma longa*) [13,14]. In recent decades, curcumin has demonstrated anti-inflammatory, antibacterial, anti-cancer, anti-Alzheimer's, anti-fungal, and other activities [15-16]. Curcumin's limited bioavailability, poor-water solubility, instability in bodily fluids, and quick metabolism have hindered its use in medical applications [17,18].

Various formulations such as liposomes, micelles, microemulsions, biopolymeric nanoparticles, nanocrystal dispersions, amorphous solids, and floating gastroretentive systems have been created to enhance the oral bioavailability of curcumin [19,20]. NPs may solve challenges related to delivering hydrophobic medications in water-based solutions, leading to a growing focus on developing drug delivery systems utilising nanoparticles. [20,21].

NPs improve the solubility and stability of active substances, broadening the possible uses of functional components, especially for environmentally sensitive substances like curcumin [21,22].

This study focused on obtaining the mixture, analysing, and evaluating NPs made of pullulan containing curcumin for applications in the biomedical area, particularly in cancer treatment. Pullulan acetate derived from modifying pullulan produced through bacterial fermentation with *Aureobasidium pullulans* strain was utilised as a biopolymeric matrix. Curcumin-loaded nanoparticles were created using nanoprecipitation and characterised for entrapment efficiency, size, and polydispersity index using spectrophotometric and dynamic light scattering methods. The study on curcumin release was conducted in phosphate-buffered saline 0.1M at pH 5 and pH 7.4. The NPs' cytotoxicity *in vitro* was assessed on different cell lines including NHA astrocytes, human fibroblasts, human glioblastoma U87 MG, and colon cancer line Caco2.

2. Materials and Methods

2.1. Materials

Curcumin, pyridine, acetic anhydride, and Pluronic F127 were purchased from Sigma-Aldrich Co. (Merck Group, Darmstadt, Germany). Acetone was purchased from S.C. AdraChim S.R.L (Bucharest, Romania). Human glioblastoma U87 MG (ATCC-HTB-14), NHA astrocytes (Lonza), colon cancer line Caco2 (ATCC-HTB-37) and human fibroblasts (ATCC-PCS-201-012) were acquired from Lonza and from ATCC. Fetal equine serum (FES), Eagle's minimum essential medium (EMEM), a mixture of penicillin, neomycin, streptomycin dissolved in 0.9% NaCl (PSN), 0.25% trypsin-ethylene-diamine-tetraacetic-acid (EDTA) solution, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F12 Glutamax), N-2 Supplement, Fibroblast Growth Medium, and Supplement Mix were acquired from Sigma-Aldrich Co. (Merck Group, Darmstadt, Germany).

Pullulan was produced through a fermentation process by *Aureobasidium pullulans ICCF 36* strain from Collection of Microorganisms of INCDCF-ICCF using glucose 8%, $NaNO_3$ 0,2%, $(NH_4)_2SO_4$ 0,2%, KH_2PO_4 0,5%, NaCl 0,2%, $MgSO_4*7H_2O$ 0,08%. As previously mentioned, pullulan was chemically changed with pyridine, dimethylformamide, and acetic anhydride to produce pullulan acetate, which had an acetyl group content of 1.86 and a degree of substitution ranging from 33 to 34% [23].

2.2. Preparation of Nanoparticles Based on Pullulan Loaded with Curcumin

Curcumin-loaded NPs were created using pullulan by nanoprecipitation. A solution containing polymer and curcumin in acetone (10:1 polymer to drug ratio) was slowly added drop by drop to an aqueous solution of Pluronic-F127 (1%). The mixture was agitated at 700 rpm at room temperature using a magnetic stirrer until the organic solvent had evaporated. The suspension was centrifuged at 11000 rpm for 25 minutes at 4°C. The nanoparticles precipitate was removed from the supernatant, which contained free curcumin, and then dispersed again in double-distilled water (sample labelled NP_PA@C).

2.3. Characterization of Nanoparticles Based on pullulan Loaded with Curcumin

The chemical interaction was analysed using Fourier-Transform Infrared (FTIR) spectra, obtained in the range of 4000 to 400 cm-1 on a Bruker Tensor 27 spectrometer (Bruker Corporation Optik GmbH, Bremen, Germany).

Particle size and polydispersity index were measured by dynamic light scattering (DLS) with a Beckman-Coulter-N4-PCS-Submicron particle analyzer Paris, France. The diluted samples were measured using double-distilled water (1:50) at a constant angle of 90 degrees and at room temperature. Ten runs were conducted for each measurement.

The entrapment efficiency of curcumin in pullulan nanoparticles was indirectly assessed using equation 1 and a UV/VIS spectrophotometer (Jasco V-630, Portland, OR, USA). The supernatant was diluted with methanol in a ratio of 0.25:10. The absorbance was measured at maximum wavelenght of curcumin in methanol 421 nm using a standard curve ($y = 0.0072 + 0.1137\ x$; $R^2 = 0.99241$).

$$EE = \frac{Curcumin\ Initial\ Concentration - Free\ Curcumin\ Concentration}{Curcumin\ Initial\ Concentration} \times 100 \quad (1)$$

2.4. Release study

Curcumin release from pullulan NPs was tested *in vitro* using the dialysis bag method under optimal sink conditions. The 0.5 mL samples were enclosed in dialysis tubes with a 14000 D cut-off from Sigma-Aldrich, Merck Group, Darmstadt, Germany. They were then submerged in 100 mL of 0.1 M phosphate buffer solution (PBS) at pH 5 and pH 7.4. The study was conducted at a temperature of 37 °C, with continuous stirring at 150 rpm/min and medium replacement. The amount of curcumin released was measured at specific time points (5, 10, 15, 20, 25, 30, 45 minutes and 1, 2, 4, 5, 6, 24, 48, and 72 hours) using a UV/VIS spectrophotometer (JASCO V-630 Spectrophotometer, Jasco International Co., Ltd., Tokyo, Japan). Calibration curves for curcumin in PBS 0.1 M at pH 5, PBS 0.1 M at pH 7.4, and ethanol were used to analyse the active compounds that were released. The PBS 0.1 M pH 5 medium was selected to mimic the slightly acidic pH of tumor cells, whereas the PBS 0.1 M pH 7.4 medium was selected to mimic the pH of normal cells.

2.5. Stability

The samples were stored in a 10 mL amber-colored glass vial at 4°C for 3 months. The drug content was evaluated to asses stability at various storage intervals.

2.6. *In Vitro* Cytotoxicity and Antiproliferative Effect of NPs based on Pullulan Loaded with Curcumin

The cell viability and antiproliferative impact were assessed using the Colorimetric MTS test. The assay relies on utilising a tetrazole chemical, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazole (MTS), and an electron coupling agent, phenazine methosulfate (PMS). The cells reduce the MTS reagent into a soluble formazan compound in the culture medium. Formazan absorbance can be measured at 490 nm straight from 96-well culture plates. Human glioblastoma U87 MG (ATCC-HTB-14), NHA astrocytes (Lonza), colon cancer line Caco2 (ATCC-HTB-37), and human fibroblasts (ATCC-PCS-201-012) cell lines were cultured in 25 cm^2 flasks at 37°C in a 5% CO_2 humidified atmosphere. The culture medium used was DMEM F12 Glutamax and N-2 Supplement for astrocytes, Fibroblast Growth Medium, Supplement Mix for fibroblasts, and EMEM for Caco2 and U87. The media were also enriched with 10% or 20% FES (for Caco2) and 1% PSN. When the cultures

reached a confluence of 75% (around 48 hours of cultivation), they were harvested by treating them with trypsin - EDTA (2 mL/vial) to detach the cell monolayer. The trypsin was then neutralised with fetal bovine serum (2 mL/vial), and the cells were homogenised by gentle pipetting. The cell suspension was then harvested into 15 mL centrifuge tubes and the cells were pelleted by centrifugation at 1200 rpm for 10 minutes. Cells were then resuspended in culture medium and adjusted to 10^6 cells/mL. 96-well plates were inoculated at a density of 8,000 cells/well. After 24 hours, the culture medium was replaced with fresh medium (180 µL/well). To assess the cytotoxicity of NP based on pullulan loaded with curcumin, astrocytes and human fibroblasts were incubated in the presence of the pullulan-based NP loaded with curcumin, for 24 hours at 37^{o}C in an atmosphere with 5% CO_2, at concentrations of 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL made in the medium, after which cell viability was determined by a colorimetric method using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, USA). After 24 hours of exposure to the specified chemical concentrations, the culture medium was substituted with 100 microL/well MTS reagent, which was diluted 1:10 with fresh medium. The cells were incubated for 3 hours in a 5% CO_2 incubator without light exposure. Subsequently, their optical densities were assessed at 495 nm using a Microplate Reader (Chameleon V Plate Reader, LKB Instruments). The optical densities were measured and compared to the values of the control (untreated cells), which were regarded as the peak values of cell viability. The antiproliferative effect of pullulan-based nanoparticles loaded with curcumin was assessed by applying the same protocol to U87 MG and Caco2 tumour cell lines. Cell viability was determined after at least one cycle of division (population doubling interval), 24 and 48 hours after exposure to the nanoparticles. Untreated cells were utilized as a control.

2.7. Statistical analysis

All experiments were made in triplicate. Results were displayed as mean value ± SD. The results were considered statistically significant if the probability is less than 5%.

3. Results and discussions

3.1. Characteristics of Nanoparticles based on Pullulan Loaded with Curcumin

Curcumin-loaded polymeric nanoparticles were produced by the nanoprecipitation technique. The nanoprecipitation approach is cost-effective, straightforward, and involves separate stages. The process entailed the precipitation of pullulan acetate in an aqueous solution with a stabiliser (Pluronic F127) to prevent nanoparticle aggregation.

Spectra were obtained to test the chemical interaction between the two components (Fig. 1). Distinct characteristic vibrations were identified for curcumin during FTIR analysis at specific wavenumbers: 3512 cm^{-1} for phenolic O-H stretching vibration, 1629 cm^{-1} for C=C stretching within its aromatic

moiety, 1510 cm^{-1} for C=O stretching vibrations, and 1026 cm^{-1} for C-O-C stretching vibrations [24]. The study of pullulan showed distinct vibrations at 3487 cm^{-1}, which are associated with the stretching vibration of hydroxyl groups (-OH) found in the glucose units and water molecules. A signal at 2956 cm^{-1} was observed, suggesting stretching vibrations from carbon-hydrogen bonds (C-H) in the glucose units [25].

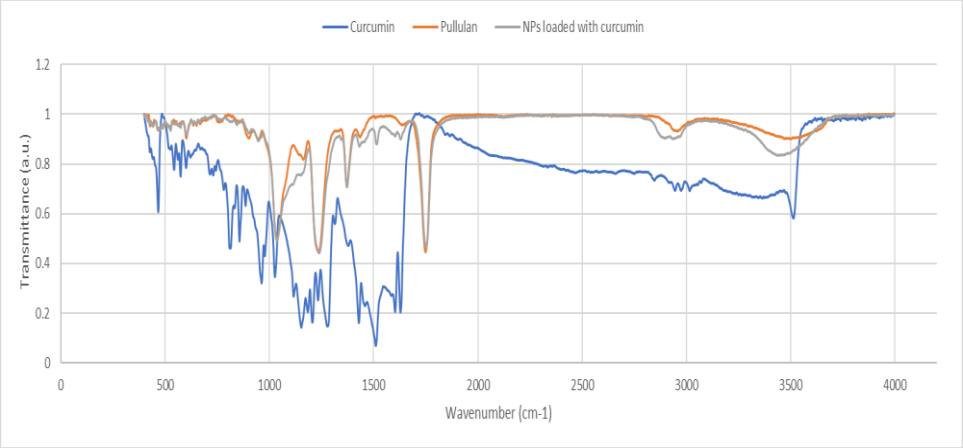

Fig. 1. IR spectra for pullulan, curcumin and NPs loaded with curcumin;

Size and homogeneity are crucial characteristics of nanocarriers that impact their circulation half-life and cellular absorption. Homogeneity is determined by calculating the polydispersity index. Systems with a polydispersity index below 0.1 are termed monodisperse, whereas those with values above 0.7 are classified as polydisperse. The DLS approach was used to determine the particle size and polydispersity index of nanoparticles containing curcumin. The nanoparticles had a size of 259.1 ± 0.26 nm, and the polydispersity index values were below 0.25, indicating considerable homogeneity. The nanoprecipitation approach works well with hydrophobic trapping chemicals and polymers that dissolve in organic solvents that are miscible with water. In our case curcumin and polymer are both hydrophobic; they dissolve in the organic solvent acetone but not in the non-solvent system, water. When the polymer solution is added to the non-solvent, the polymer rapidly desolves, resulting in the instantaneous production of polymeric nanoparticles. The polymer precipitates, causing instantaneous drug entrapment, as soon as the solvent containing the polymer has diffused into the dispersing medium. The Marangoni effect, which results from interfacial turbulences at the solvent-non-solvent interface, controls the rapid production of nanoparticles as explained by Gref et al. [26]. The process had a good entrapment efficiency (EE) of 87.46 ± 0.12%.

Our results align with previous studies. For instance, pullulan acetate nanoparticles containing epirubicin had sizes ranging from 185.7 nm to 423 nm and drug entrapment values of up to 64.8±1.7% [27]. Another study involved pullulan acetate nanoparticles enclosing lopinavir, with a size of 197 nm and an entrapment efficiency of around 75% [28]. Additionally, paclitaxel-loaded

pullulan acetate nanoparticles had a size of 253.0 ± 65.5 nm and a drug content of 13.1% [29].

It can be seen in Fig. 2 the stability of curcumin-loaded nanoparticles was evaluated by storing them in amber glass vials at 4 °C for three months.

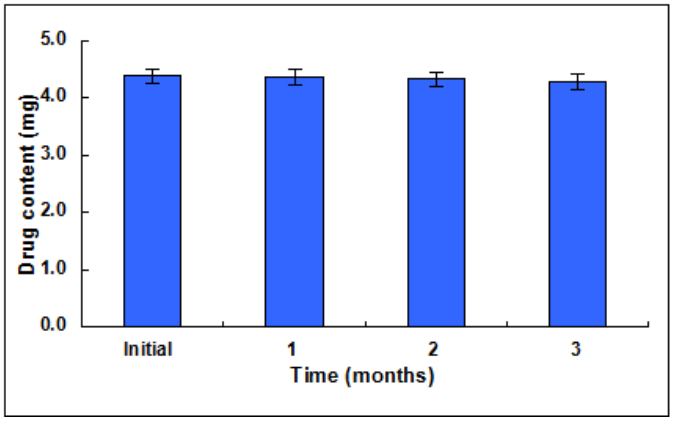

Fig. 2. Stability of nanoparticles based on pullulan loaded with curcumin at 4 °C

The drug content was measured at various time points (initial, 1, 2, 3 months). The nanoparticles showed excellent stability at 4 °C, maintaining nearly the same amount of encapsulated curcumin after one month (drug loss < 0.5%) and after three months (drug loss < 2%), with no aggregation seen during storage.

3.2. Release profiles of curcumin

Fig. 3 shows the release profiles of curcumin from nanoparticles in PBS 0.1 M pH 7.4 compared to PBS 0.1 M pH 5, at 37 °C under continuous shaking (100 rpm/min). The samples showed a slight burst phenomenon, probably explained by the release of curcumin crystals located on the surface of pullulan nanoparticles (24.78% for PBS 0.1 M pH 7.4, and 18.70% for PBS 0.1 M pH 5 after 30 min). The nanoparticles showed a cumulative drug release value of 48.30% for PBS 0.1 M pH 7.4, and 35.30% for PBS 0.1 M pH 5 after 6 h, respectively. The nanoparticles showed a higher release rate of curcumin in neutral medium than in slightly acidic medium with a maximum value of curcumin release reached after 72 hours (78.02 % for PBS 0.1 M pH 7.4, and 60.09 % for PBS 0.1 M pH 5).

The drug release mechanism from the curcumin loaded nanoparticles was studied by analysing the experimental data with various kinetic models including zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Hixson-Crowell models. The correlation coefficient (R^2) was utilised to compare the models, with a value around 1 indicating a stronger association. Based on R^2 values (Table 1), the release of the curcumin from pullulan NPs was best described by the Korsmeyer-Peppas (R^2 = 0.9671 for release in PBS pH 7.4 and R^2 = 0.9516 for release in PBS pH 5) and Higuchi models (R^2 = 0.9568 for release in PBS pH 7.4 and R^2 = 0.9645 for release in PBS pH 5).

Korsmeyer-Peppas and Higuchi release models are presented in equation 2 and 3:

$$M(t)/M(\infty) = k_{KP} \times t^{n} \quad (2)$$

$$M(t)/M(\infty) = k_{H} \times t^{1/2} \quad (3)$$

where $M(t)/M(\infty)$ is a fraction of drug released at time t, $M(t)$ represents drug released at time t and $M(\infty)$ represents the total amount of drug located in the carrier; k_{KP}, k_H are Korsmeyer-Peppas and Higuchi constants and n is the diffusional exponent. The Korsmeyer-Peppas and Higuchi models were applied only for the first 60% cumulative release of curcumin.

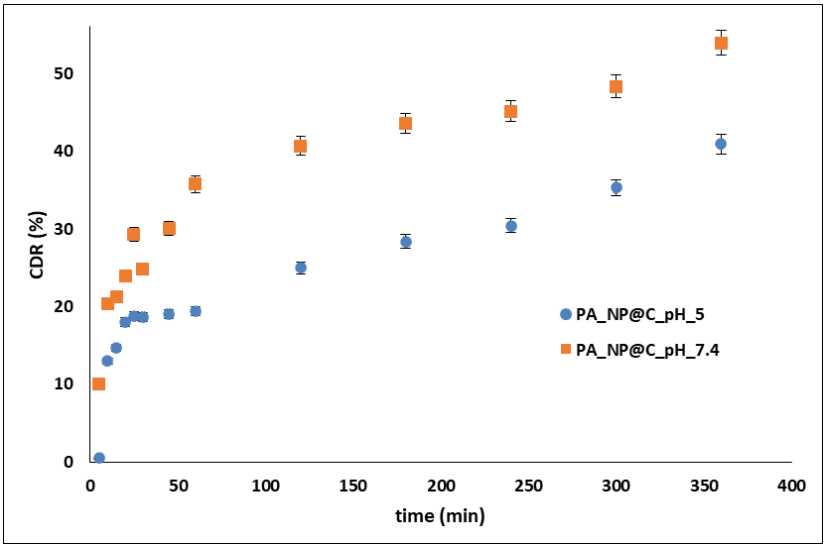

Fig. 3. Release profiles of curcumin from pullulan nanoparticles (PA_NP@C) in PBS 0.1 M pH 7.4 compared to PBS 0.1 M pH 5

Table 1.

Correlation Coefficient Values (R^2)

Model	Curcumin release in PBS 0.1 M pH 7,4	Curcumin release in PBS 0.1 M pH 5
	R^2	R^2
Zero Order	0.8915	0.8268
First Order	0.9256	0.8737
Hixson-Crowell	0.9152	0.8574
Higuchi	0.9568	0.9645
Korsmeyer-Peppas	0.9671	0.9516

Table 2.

Parameters of Korsmayer-Peppas and Higuchi models

Release medium	n	k_{KP}	k_H
Curcumin release in PBS 0.1 M pH 7.4	0.2654	32.71	15.33
Curcumin release in PBS 0.1 M pH 5	0.2755	21.92	11.63

The Korsmeyer-Peppas model uses a constant, kKP, which is influenced by the carrier's features, and a diffusional exponent, n, that indicates the release mechanism's nature. For n values below 0.45, release is primarily controlled by the Fickian diffusion mechanism. When n ranges from 0.45 to 0.89, the release exhibits anomalous diffusion behaviour (non-Fickian diffusion). For n values over 0.89, the release is guided by a super-case-II transport mechanism [30]. In Higuchi's model, kH is a constant that is proportionally related to the release rate during the burst stage of the delivery process. Table 2 displays the constants kKP and kH, as well as the diffusional coefficient n values. The parameter n of the Korsmeyer-Peppas model is smaller than 0.45 for pullulan-based NP, indicating that the release from these systems is mostly controlled by Fickian diffusion mechanism.

3.3. Cytotoxycity

Tumor cells exhibit unregulated cell proliferation [31], necessitating the regulation of cell differentiation to manage tumor growth.

Evaluating the cytotoxic effects of curcumin-loaded nanoparticles is crucial in determining their suitability for therapeutic use. *In vitro* studies were done to evaluate the cytotoxic effects of curcumin-loaded nanoparticles on different cell types. The research assessed cell survival and proliferation after exposure to varying doses of curcumin-loaded nanoparticles for 24 and 48 hours.

In Fig. 4 is presented the cytotoxicity of pullulan-based NP loaded with curcumin on NHA astrocytes and human fibroblasts cell lines, while the Fig. 5 displays the antiproliferative effect of the same formulation on tumor cell lines human glioblastoma U87 MG and colon cancer line $CaCo_2$.

The *in vitro* experiments demonstrated that polymeric nanoparticles made of pullulan and containing curcumin did not have a negative impact on NHA astrocytes and human fibroblasts. The polymer used to make the nanoparticles may have had a substantial impact on their cytotoxicity. The selection of material can influence the biocompatibility and safety of the nanoparticles. The cytotoxic effects of curcumin-loaded nanoparticles may differ based on the specific type of cells they are designed to affect. Cancer cells have greater sensitivity to pullulan-based nanoparticles containing curcumin compared to normal cells, providing a significant benefit for cancer treatment. Previous research has described the non-toxic nature of curcumin in healthy cells [32,33]. The lack of cytotoxicity effect on normal cells can be attributed to the slow release of pullulan-based nanoparticles containing curcumin.

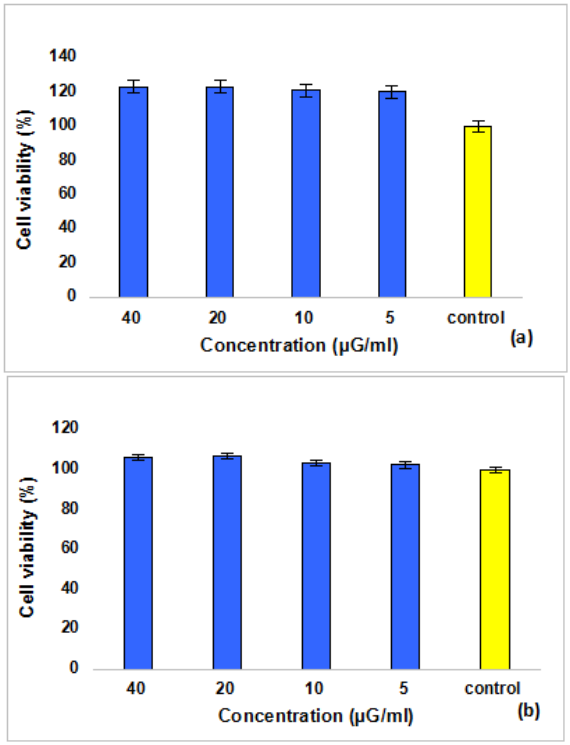

Fig. 4. Cytotoxicity of NP based on pullulan loaded with curcumin on NHA astrocytes (a) and human fibroblasts (b)

The period of time exposed to curcumin-loaded nanoparticles affects the anti-proliferative impact. Fig. 5 demonstrated that extended exposure to high concentrations might enhance the toxicity of tumor cells, resulting in an antiproliferative effect after 48 hours, although shorter exposure periods of 24 hours may be less detrimental. The quantity of curcumin included into the nanoparticles can impact the survival of cancer cells. At a dosage of 40 ug/mL after 48 hours, nanoparticles containing curcumin displayed the most notable antiproliferative impact on human glioblastoma U87, as demonstrated in Fig. 5. The colon cancer cell line Caco2 showed the greatest decrease in cell viability when exposed to nanoparticles containing curcumin at a dosage of 20 ug/mL. This observation indicates that the response to curcumin-loaded nanoparticles can differ among various cancer cell types.

These findings suggest that the decline in the viability of cancer cells was dose- and time-dependent.

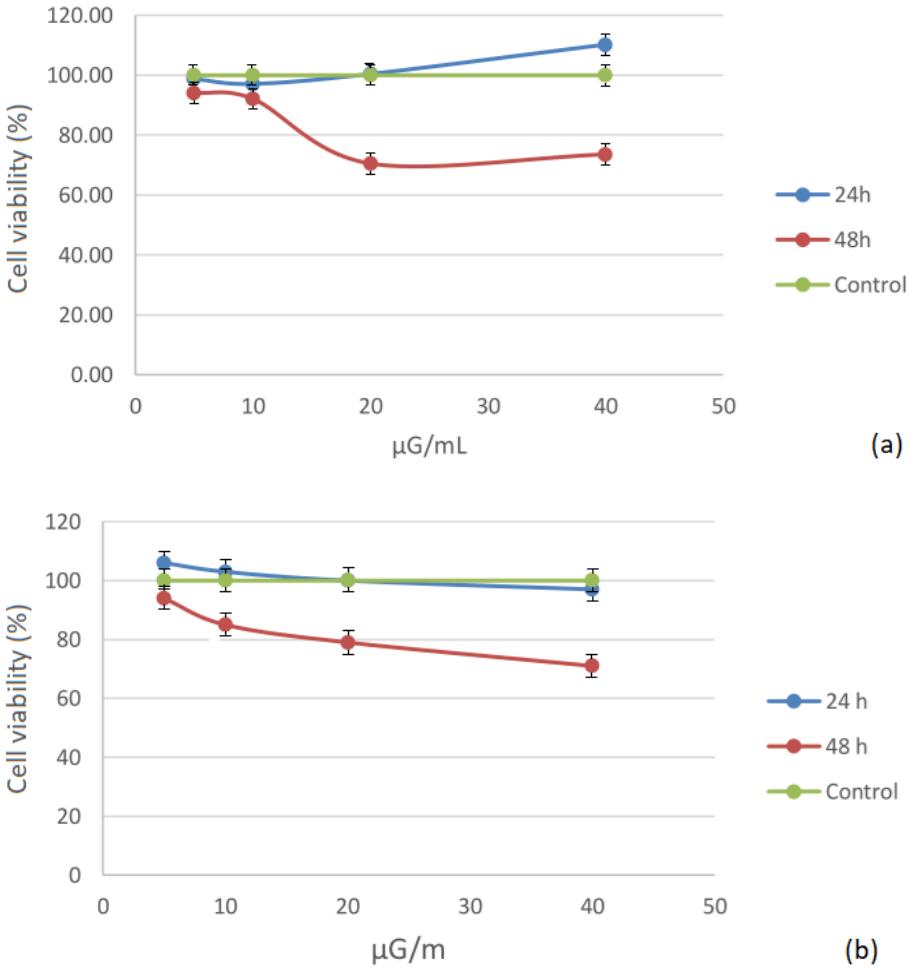

Fig. 5. Antiproliferative effect of NP based on pullulan loaded with curcumin on colon cancer line $CaCo_2$ (a) and human glioblastoma U87 MG (b)

4. Conclusions

Pullulan-based polymeric nanoparticles loaded with curcumin were successfully produced by nanoprecipitation. The nanoparticles exhibited nanometer-scale dimensions, uniform distribution, entrapment efficiency over 80%, and remained stable for three months at 4 °C. The nanoparticles exhibited a faster release of curcumin in a neutral environment compared to a slightly acidic environment, with the highest amount of curcumin released after 72 hours. The in vitro investigations demonstrated that polymeric nanoparticles did not have a negative impact on NHA astrocytes and human fibroblasts. Prior to clinical application in treating diseases such as cancer, it is essential to thoroughly assess the cytotoxic effects of curcumin-loaded nanoparticles through preclinical research to guarantee their safety and efficacy. The described characteristics of the nanoparticles obtained render them valuable for a range of biomedical uses. Polymeric nanoparticles, due to continuous scientific research and technical

advancements, are expected to play a significant role in the future pharmaceutical industry by introducing innovative drug delivery technologies.

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R E F E R E N C E S

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