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Regiane G. de Lima

UNESP: Universidade Estadual Paulista Julio de Mesquita Filho

Maria T. Barros

Universidade Nova de Lisboa

Maria M. Cardoso

Universidade Nova de Lisboa Faculdade de Ciências e Tecnologia: Universidade Nova de Lisboa
Faculdade de Ciências e Tecnologia

wilson R. Cunha

Unifran: Universidade de Franca

Elisa. G. Lucas

Unifran: Universidade de Franca

Pedro S. S. R. Cavallari

Unifran: Universidade de Franca

Pedro V. Baptista

Universidade Nova de Lisboa Faculdade de Ciências e Tecnologia: Universidade Nova de Lisboa
Faculdade de Ciências e Tecnologia

Luis R. Raposo

Universidade Nova de Lisboa Faculdade de Ciências e Tecnologia: Universidade Nova de Lisboa
Faculdade de Ciências e Tecnologia

Alexandra R. Fernandes

Universidade Nova de Lisboa Faculdade de Ciências e Tecnologia

Marcio L. Andrade e Silva

Unifran: Universidade de Franca

Luciano Menini

IFES: Instituto Federal de Educação Ciência e Tecnologia do Espírito Santo

Luciana A. Parreira

UFES: Universidade Federal do Espírito Santo

Mario F. C. Santos

mariosantos408@gmail.com

Universidade Federal do Espírito Santo <https://orcid.org/0000-0003-1981-834X>

Rosângela S. Laurentiz

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Antiproliferative evaluation of hinokinin-loaded PLGA and PLGA-PEG nanoparticles

Regiane G. de Lima^a, Maria T. Barros^b, Maria M. Cardoso^b, Wilson R. Cunha^d, Elisa. G. Lucas^d, Pedro S. S. R. Cavallari^d, Pedro V. Baptista^c, Luis R. Raposo^c, Alexandra R. Fernandes^c, Marcio L. Andrade e Silva^d, Luciano Menini^e, Luciana A. Parreira^f, Mario F. C. Santos^{f*}, Rosângela S. Laurentiz^{a*}.

^a*Departamento de Física e Química, Universidade Estadual Paulista Júlio de Mesquita Filho, Ilha Solteira-SP, 15385-000, Brazil.*

^b*LAQV-REQUIMTE, Chemistry Department, NOVA School of Science and Technology, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal*

^c*UCIBIO, Departamento Ciências da Vida, NOVA School of Science and Technology, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal*

^d*Núcleo de Ciências Exatas e Tecnológicas, Universidade de Franca, Av. Dr Armando Salles Oliveira, 201 Parque Universitário – Franca – SP CEP 14404-600, Brazil*

^e*Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, Campus de Alegre, Programa de Pós-graduação em Agroecologia Rodovia ES-482), Km 47 Distrito de Rive, Alegre - ES CEP: 29500-000, Brazil*

^f*Universidade Federal do Espírito Santo, Campus de Alegre, Centro de Ciências Exatas, Naturais e Saúde, Departamento de Física e Química, Alto Universitário s/n, Centro, Alegre – ES CEP: 29500-000, Brazil, marioantos408@gmail.com*

*Corresponding author: mariosantos408@gmail.com (Mario F. C. Santos) and rosangela.laurentiz@unesp.br (Rosângela S. Laurentiz)

ORCID:

Regiane G. de Lima– <http://orcid.org/0000-0002-2419-5848>

Maria T. Barros- <http://orcid.org/0000-0002-2307-1368>

Maria M. Cardoso– <http://orcid.org/0000-0001-7766-7557>

Wilson R. Cunha – <https://orcid.org/0000-0002-1952-1944>

Elisa. G. Lucas - <https://orcid.org/0009-0008-8690-0363>

Pedro S. S. R Cavallari-<https://orcid.org/0000-0002-8149-3032>

Pedro V. Baptista- <http://orcid.org/0000-0001-5255-7095>

Luis R Raposo- <http://orcid.org/0000-0002-8637-346>

Alexandra R. Fernandes- <http://orcid.org/0000-0003-2054-4438>

Marcio L. Andrade e Silva-<https://orcid.org/0000-0002-9442-4757>

Luciano Menini- <https://orcid.org/0000-0003-3656-5428>

Luciana A. Parreira– <http://orcid.org/0000-0001-7041-9999>

Mario Ferreira Conceição Santos – <http://orcid.org/0000-0003-1981-834X>

Abstract

Hinokinin (HNK), a dibenzylbutyrolactonic lignan from *Piper cubeba* L.f., has been reported to exhibit cytotoxicity, but its low solubility may limit bioavailability. Here, HNK was extracted and encapsulated into PLGA and PLGA-PEG nanoparticles using a modified o/w emulsification–solvent evaporation method, yielding encapsulation efficiencies of 88% and 83%, respectively. The nanosystems exhibited a spherical morphology and a mean size of 233–242 nm, with negative zeta potentials, indicating colloidal stability. In vitro release assays demonstrated a rapid release profile: >80% of HNK was released within 2 h and near-complete release after 15 h, with no major pH-dependent differences. Antiproliferative screening (24 h, MTS) in HCT116, A2780, and A549 cells revealed a concentration-dependent reduction of viability for free HNK, whereas HNK-loaded nanoparticles produced comparable or lower effects, and unloaded nanoparticles were non-toxic. Collectively, these results show that HNK can be efficiently incorporated into PLGA-based nanoparticles with rapid release and modest antiproliferative activity in vitro, warranting further mechanistic studies to elucidate the molecular basis of its antitumor effects.

Keywords: lignan; biodegradable polymer; encapsulation efficiency; cytotoxicity; burst release

Introduction

Cancer chemotherapy still faces major challenges related to systemic toxicity, limited selectivity, and reduced therapeutic efficacy, often associated with poor solubility, instability, and inadequate delivery of anticancer agents (Krasteva and Georgieva 2021). Natural products remain central to drug discovery, with lignans constituting a significant class of secondary metabolites exhibiting diverse biological activities, including antitumor, antiproliferative, and pro-apoptotic effects (Yuliana et al. 2011; Godoy et al. 2018; Cunha et al. 2012). Lignans are secondary metabolites produced by plants and perform various functions in these organisms, including defense against fungi, insects, and mechanical injuries. This diversity of biological properties in plants suggests a possible application of these compounds for controlling diseases that also affect humans and animals (Cunha et al. 2012; Marcotullio et al. 2014). The wide variety of biological properties attributed to lignans is directly related to the structural diversity of these compounds (Lima et al. 2017a; Pissurno and Laurentiz 2017; Cao et al. 2013).

Hinokinin (Figure 1) is a dibenzylbutyrolactonic lignan derived from *Piper cubeba* that has attracted attention for its reported cytotoxicity against various tumor cell lines (Lima et al. 2022a). However, comparative studies have shown that HNK may exhibit lower potency than other lignans, a limitation often attributed to its low aqueous solubility and limited bioavailability, which can compromise effective cellular exposure [6,10–12]. In a study by Pusztai et al. (2010), the HNK was also examined as an antitumor promoter in the expression of the human cytomegalovirus (HCMV) immediate-early (IE) antigen in human lung adenocarcinoma (A-549) cells. After establishing an IC_{50} of 115 μ M in an A-549 cell line, the authors found that HNK reduced IE antigen expression in HCMV-infected lung cancer cells in a dose-dependent manner (ID1: 81%; ID10: 57% reduction) (Pusztai et al. 2010).

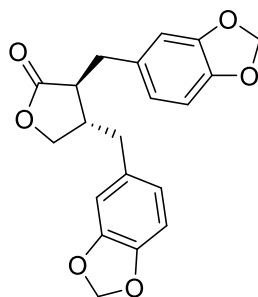


Fig. 1. Chemical structure of (-)-hinokinin.

These studies demonstrated that HNK has cytotoxic potential but is less active than several natural or synthetic compounds that have already been evaluated and described in the literature. The lower cytotoxicity of HNK may be associated with its low solubility, which reduces absorption and thus bioavailability in biological systems, as is the case with most chemotherapeutic agents in use (Crucho 2015). Given the high potential of this compound and other lignans, new methods need to be explored to bring, uptake, retain, release, activate, localize, and target drugs at the right time, period, dose, and place, optimizing their use (Lima et al. 2017b). 6,6'-dinitrohinokinin, a synthetic lignan obtained from HNK that exhibits good schistosomicidal activity, was encapsulated in PLGA nanoparticles and showed excellent therapeutic activity in vivo in comparison with the free form (Lima et al. 2017b). In another study using lignans, podophyllotoxin (PPT) was encapsulated in PLGA nanoparticles (PLGA-NPs) and evaluated against HeLa cells. The concentration yielding the highest inhibition of cell viability was 96 μ M, causing 70% inhibition with PLGA-PPT-NPs and only 30% with free PPT at the same concentration. In this case, encapsulation increased the percentage of cell growth inhibition relative to the free compound (Silva et al. 2005).

Therefore, nanoparticle-based drug delivery systems, particularly those based on biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA) and PLGA-PEG, have been widely investigated as strategies to improve the solubility, stability, and pharmacological performance of poorly soluble compounds (Rezvantab et al. 2018). In parallel, molecular docking has been increasingly employed to identify potential intracellular targets and to guide mechanistic hypotheses (Marcotullio et al. 2014). Aurora B kinase, a central component of the chromosomal passenger complex and a critical regulator of mitosis, is frequently overexpressed in cancer cells and represents a validated therapeutic target (Enders and Milovanovic 2014; Saraiva et al. 2008; Pusztai et al. 2010).

In this context, the present study aimed to encapsulate HNK within PLGA and PLGA-PEG nanoparticles, evaluate its antiproliferative activity across different tumor cell lines, and, through molecular docking, explore the interaction of HNK with Aurora B kinase.

Material and methods

Materials

P. cubeba dried fruits were imported from the Floral Seed Company (LOTE No. 200817234), Dehradun, India. Extractions, purifications, and reactions were monitored by thin-layer chromatography (TLC) using Merck 60 F254 silica gel plates and visualized by UV detection with a BOITTON 254 nm and 365 nm UV-emitting lantern, or by sulfuric vanillin solution, followed by hot plate development. Nuclear magnetic resonance spectra (^1H and ^{13}C NMR) were obtained on a Bruker DRX-400 and a 500 MHz spectrometer, both located at the Nuclear Magnetic Resonance Laboratory of the Department of Chemistry at the Ribeirão Preto School of Philosophy, Sciences and Letters (USP). Chemical shifts (δ) are reported in parts per million (ppm). A SHIMADZU CBM-20A binary system chromatograph, Prominence-LC-6AD, equipped with a manual injector with a 20 μL or 500 μL loop, a DGU-20A5 degasser coupled to a DAD model SPD-M20A detector and a UV-Vis model SPD-20A detector with data acquisition via a microcomputer, and a SHIMADZU Shim-pack ODS analytical column, 250 x 4.20 mm, 5 μm , equipped with pre-columns, and a SHIMADZU Shim-pack ODS semi-preparative column, 250 x 20 mm, 5 μm , equipped with pre-columns, were used. All reagents and solvents were purchased from commercial sources (Alpha Aesar, Sigma-Aldrich, and Synth) and used without further purification. Preformed PLGA copolymers with the lactic acid: glycolic acid ratio: 65:35 ($M_w = 45000 - 70000$), and 10% PLGA-PEG-diblock ($M_w \sim 600000$) Resomer® RGP d 50105 all purchased from Sigma-Aldrich were used. Mowiol® 18-88 surfactant was purchased from Sigma Aldrich ($M_w = 130000$).

Lignan extraction

Hinokinin (HNK) was isolated from dried, powdered *Piper cubeba* fruits using the method described by Silva et al. (2005). Briefly, 2.0 kg of powdered material, dried in an air-circulation oven at 40 °C and milled in a knife mill (Marconi), was subjected to exhaustive maceration in 6 L of ethanol at room temperature for 7 days. After filtration and vacuum concentration, the crude ethanolic extract (EBE) was subjected to liquid-

liquid partitioning by suspending 200 g of solvent-free EBE in hydromethanolic solution (MeOH/H₂O, 9:1, v/v) and partitioning against n-hexane (7 × 250 mL), yielding the hexane fraction and the hexane-methanol fraction (HMF, 45 g), the latter enriched in lignans. The HMF was fractionated by vacuum liquid chromatography (VLC) on silica gel (600 g) using a polarity gradient from hexane to ethyl acetate, and fractions were monitored by thin-layer chromatography (TLC) with vanillin-sulfuric acid as a visualizing agent. Sixteen subfractions were collected, and subfraction 9 (9.60 g), eluted with hexane/DCM (6:4–1:1, v/v), was further purified by classical column chromatography (CCC) on silica gel (220 g) with a hexane/ethyl acetate gradient, yielding 12 subfractions. HPLC-DAD analysis of subfraction 9 revealed three major peaks at retention times of 18.094, 21.413, and 35.535 min (254 nm). Subfraction 9-7 (1.15 g), eluted with hexane/ethyl acetate (7:3, v/v), showed a single dominant peak at *tr* = 21.456 min (254 nm), indicating high purity (Fig. 14). Purity was verified by analytical HPLC prior to spectroscopic analysis, and the structure of HNK was confirmed by ¹H and ¹³C NMR spectroscopy. NMR spectra (supplementary material) were recorded on a Bruker DRX-400 spectrometer (300 MHz for ¹H NMR and 75 MHz for ¹³C NMR) (Enders and Milovanovic, 2014).

Preparation of PLGA and PLGA-PEG nanoparticles

HNK was incorporated into PLGA and PLGA-PEG nanoparticles (NPs) using a modified emulsification solvent evaporation technique o/w (oil/water) (Lima et al. 2022b). PLGA or PLGA-PEG (40 mg) were mixed in the ratio of 10:1 polymer: HNK (in 4 ml of dichloromethane). This solution was then added to 6.25 ml of a 2.2% (w/v) solution. In a single aliquot under stirring for 5 min at approximately 112 ×g (radius of 8.5 cm and 1086 rpm) and sonicated for 90 seconds at 70% A using a ½” tip (Sonifer® Branson SFX550). The suspension of the resulting NPs was stirred at room temperature for 24 h to ensure complete evaporation of the organic solvent. The NPs produced were separated by centrifugation (15 min at 22,200 ×g with a radius of 8.5 cm and 15284 rpm) and washed three times with Millipore water. The obtained NPs were frozen in liquid nitrogen, lyophilized at -55 °C and 0.5 kPa. and stored at -10 °C until use. Unloaded NPs were prepared similarly without adding HNK to the polymer solution. Figure 2 illustrates the scheme of synthesis of HNK loaded NPs.

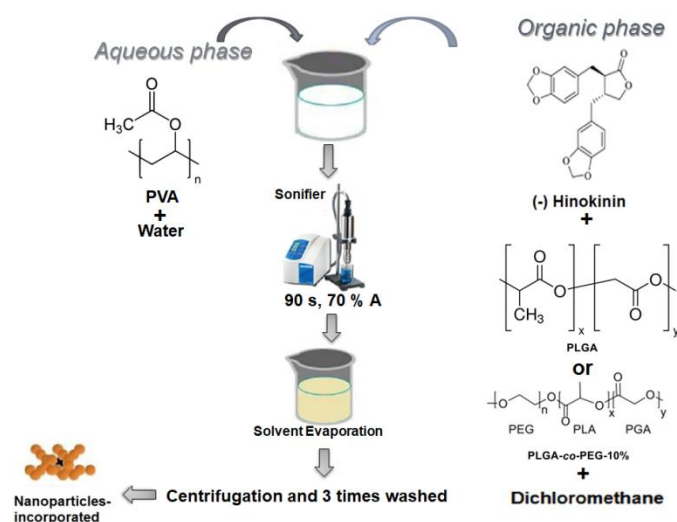


Fig. 2. Scheme of (-)-hinokinin loaded PLGA NP (PLGA-HNK) and (-)-hinokinin loaded PLGA-PEG NP (PLGA-PEG-HNK) synthesis

Particles size and zeta potential

Average particle size and particle size distribution were measured by dynamic light scattering (DLS) using a light scattering instrument at a 90° scattering angle (Horiba Scientific Nano Particle Analyzer SZ-100). The refractive index was 1.330, and the temperature was kept at 25 °C during the measurements. The weighted-average intensity was calculated as the average of three measurements. The lyophilized nanoparticles were dispersed in Mili-Q water. NPs zeta potential (ξ) measurements were determined by the laser Doppler electrophoresis technique (Horiba Scientific Nano Particle Analyzer SZ-100). The zeta potential (ξ) measurements reflect the surface potential of the particles for checking the stability of the systems (Lima et al. 2022b)

NPs Morphology - Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The morphological evaluations of the NPs were performed using a scanning electron microscope (SEM) (JEOL 7001F FEG-SEM - FIB – Zeiss) and a transmission electron microscope (TEM) (Hitachi H8100 TEM). SEM images were obtained at a 5000× magnification. The lyophilized NPs were deposited on a metal stub on which a thin film of a conductive metal was sprayed onto the surface of the samples. A drop of aqueous nanoparticle suspension (0.1 mg NP / ml) was deposited on the carbon-coated copper grid for visualization by TEM. For each sample, the mean diameter was calculated

from measurements of 100 randomly selected particles using the ImageJ Program (Schneider et al. 2012).

Differential Scanning Calorimeter (DSC)

This technique was used to characterize the physical state of lignans in nanoparticles and to elucidate any interactions between lignans and polymers. For these analyses, sealed alumina pans were used, with a temperature range of 25-500 °C and a heating rate of 10 °C/min (Koopaei et al. 2012; Fattahi et al. 2018) in a Netzsch TGA-DSC-STA 449 F3 Jupiter.

Encapsulation efficacy

The encapsulation efficiency was determined by measuring the absorbance at 288 nm of a solution of lignan-containing NPs (3 mg NPs in 3 ml dichloromethane) and comparing it with a lignan calibration curve (absorbance $\times$ concentration) at five different concentrations. The encapsulation efficacy was expressed as a percentage according to the formula: total amount of lignan determined in NPs (by absorbance) $\times$ 100 / total amount of lignan used in NPs production (Enders and Milovanovic 2014).

***In vitro* HNK cumulative release studies**

In order to study the lignan release profiles, *in vitro* tests were carried out in an aqueous medium at pH = 1.2, simulating gastric fluid, and in a phosphate buffer solution (PBS) at pH 7.4, simulating intestinal fluid, for a maximum of 12 h, and also close to blood pH (6.8 to 7.2). NPs-HNK (5mg) were added to 200 ml of each solution (pH 1.2 or 7,4) in a tightly sealed vial. The vials were placed in a shaker set to 160 $\times$ g and 37.0 °C. At time intervals, aliquots were taken from each vial. To keep the particle mass/volume ratio constant throughout the release assay, 1 ml of fresh medium was added for each 1 ml of sample collected. To extract the lignans from the aqueous medium, each 1 ml aliquot was mixed with 800 μ L of CH₂Cl₂ by vortexing. The organic fraction of this mixture was removed using a syringe and analyzed with a UV-Vis spectrophotometer. The absorbance value obtained was compared to a pure lignan calibration curve (absorbance $\times$ concentration) (Margarida et al. 2016).

Cell culture

HCT116, A2780, and A549 cancer-derived cell lines (from human colorectal and ovarian carcinomas and lung adenocarcinoma, respectively) were cultivated in Dulbecco's modified Eagle medium (DMEM) (HCT116 and A549) or RPMI 1640 medium (A2780). All media were supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% PenStrep solution (both from Thermo Fisher Scientific, Waltham, MA, USA). Cell cultures were grown at 37.0 °C, in an atmosphere with 5.0% (v/v) CO₂ and high humidity, as previously described (Maroń et al. 2018; Sutradhar et al. 2019). A2780 cancer cell line was purchased from Merck (Darmstadt, Germany) and HCT116 and A549 cancer cell lines from American Type Culture Collection (ATCC, Manassas, VA, USA).

Cell Viability assays

Cell viability was determined in 96 well-plates with 7500 cells per well. After a first incubation of 24 h under the conditions described above, the culture media were replaced with Dimethyl sulfoxide (DMSO) (vehicle control), Lignan, NPs-Lignan, or Hinokinin (HNK) stock solution in DMSO. Cell cultures were incubated for 24 h, and cell viability was determined using the CellTiter 96® Aqueous Non-Radioactive Proliferation assay (MTS assay) (Promega, Madison, WI, USA) (Maroń et al. 2018; Sutradhar et al. 2019). The metabolically active cells possess dehydrogenases that reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), together with the electron coupling reagent phenazinemethosulfate (PMS), to form formazan. The amount of formazan formed is directly proportional to the number of viable cells, and its absorbance can be measured at 490 nm in a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). In all biological replicates, internal controls are performed using doxorubicin (IC₅₀ 0.4 µM) in HCT116 cells. All viability data is normalized to each cell type incubated with the same % of DMSO as in free HNK.

Results and Discussion

HNK extraction

HNK was extracted from *P. cubeba* fruits at a yield of 1.5%, based on a 100 g mass of crude extract subjected to silica gel column purification. The structure of HNK was confirmed by ¹H and ¹³C NMR spectra. The results obtained are in agreement with those published by Silva et al. (2005) (Yadav et al. 2014).

Morphology study of PLGA nanoparticles (PLGA-NPs)

The surface morphology and size of PLGA-NPs, PLGA-HNK-NPs, PLGA-PEG-NPs, and PLGA-PEG-HNK-NPs were examined by SEM, and the obtained images are shown in Figure 3A-D, respectively, showing that all the nanoparticles have a regular spherical shape and a smooth surface. The particle size distribution, determined by DLS based on intensity (Table 1), is consistent with the particle sizes observed from the SEM micrographs shown in Figure 3. Nanoparticles exhibited low polydispersity, indicating a narrow particle-size distribution in the solution. Particles with a PDI of 0.1 to 0.4 are considered to have a medium polydispersity, and those above 0.4 are considered very polydisperse (Bhattacharjee 2016).

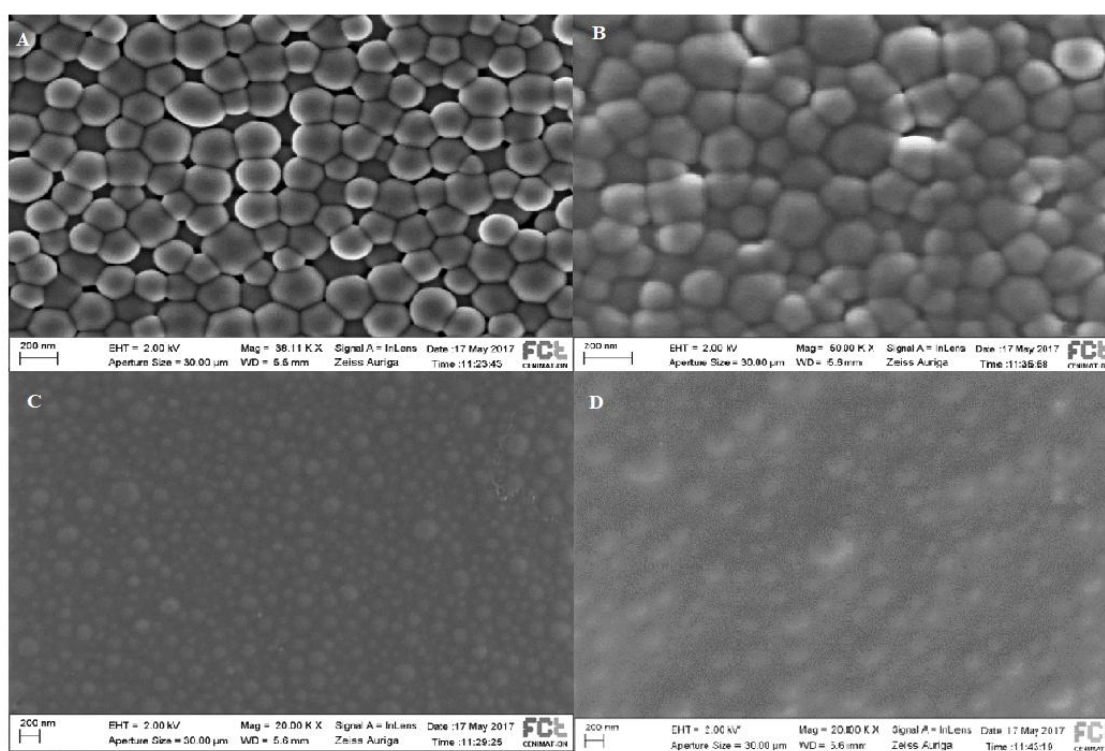


Fig. 3. Micrographs of NPs obtained by SEM. (A) PLGA-NPs, (B) PLGA-HNK-NPs, (C) PLGA-PEG-NPs and (D) PLGA-PEG-HNK-NPs

Table 1. Average particle size, polydispersity index and zeta potential results of PLGA-NPs PLGA-HNK-NPs, PLGA-PEG NPs and PLGA-PEG-HNK-NPs.

Particles	Average Particle Size (nm)	P.D.I.	Zeta ξ (mV)
PLGA-NPs	242 $\pm$ 30	0.087	-53.7 $\pm$ 0.1
PLGA-HNK-NPs	238 $\pm$ 10	0.116	-30.1 $\pm$ 0.5
PLGA-PEG-NPs	233 $\pm$ 50	0.146	-69.5 $\pm$ 0.7

PLGA-PEG-HNK-NPs	240 ± 20	0.119	-114.3 ± 0.6
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The zeta potential of PLGA-NPs, PLGA-HNK-NPs, PLGA-PEG-NPs and PLGA-PEG-HNK-NPs measured at neutral pH, presented in Table 1, indicated negatively charged surfaces, due to the presence of carboxylic groups in the PLGA structure (Bhattacharjee 2016). For all nanoparticles produced, a high zeta potential was observed, indicating good stability of the nanoparticle systems due to effective electrostatic particle between particles (Peça et al. 2012). The values of zeta potential for PLGA-NPs and PLGA-PEG-NPs vary widely depending on the particle size, the synthesis conditions, and the embedded molecule (Al Meslmani et al. 2017; Pieper and Langer 2017). In Figure 4, micrographs of nanoparticles obtained by TEM show nanoparticles with spherical shapes, however, very crowded, which could influence the stability analysis (Gholizadeh et al. 2018).

Differential Scanning Calorimetry (DSC) is a technique used to measure the temperature and enthalpy associated with phase transitions in materials as a function of temperature and time. Thermograms exhibited in Figure 5 were made for comparison between PLGA-NPs, PLGA-PEG-NPs, PLGA-HNK-NPs, PLGA-PEG-HNK-NPs and HNK. A glass transition of the polymer PLGA (at 48 °C)(Fattahi et al. 2018) was observed in all NPs. The observed endothermic peak at 83 °C confirms that HNK is in the structure of the PLGA-HNK-NPs and PLGA-PEG-HNK-NPs.

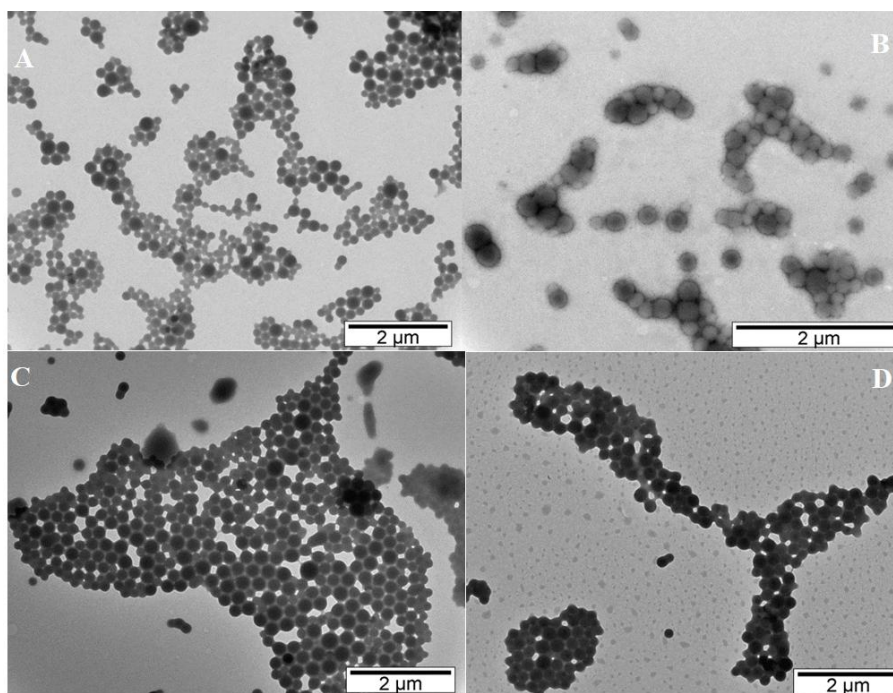


Fig. 4. Micrographs of nanoparticles obtained by TEM. (A) PLGA-NPs, (B) PLGA-HNK-NPs, (C) PLGA-PEG-NPs and (D) PLGA-PEG-HNK-NPs

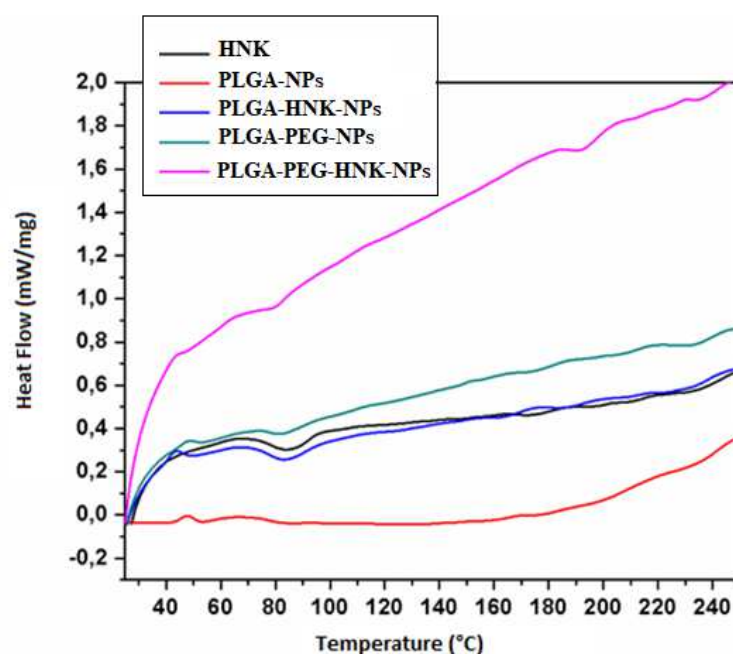


Fig. 5. PLGA-NPs, PLGA-HNK-NPs, PLGA-PEG-NPs, PLGA-PEG-HNK-NPs and HNK thermograms

Encapsulation efficiency

The encapsulation efficiency of HNK was evaluated for PLGA-HNK-NPs and PLGA-PEG-HNK-NPs. Analyses were performed as previously described using a variable-wavelength UV detector and selecting the absorbance at the wavelength of maximum absorbance (288 nm), as shown in Figure 6. The concentration of HNK was determined using the HNK calibration curve obtained (Figure 6). The percentage of encapsulated HNK in PLGA-NPs and PLGA-PEG-NPs was determined by the equation: encapsulation efficiency (%) = total amount of determined HNK in nanoparticles $\times$ 100 / total amount of HNK used in NPs preparation. The encapsulation efficiencies obtained were 88% for PLGA-HNK-NPs and 83% for PLGA-PEG-HNK-NPs. Comparing the two nanoparticles analyzed, the lower percentage observed for PLGA-PEG-NPs may be due to the presence of PEG, which increases NP hydrophilicity and thus reduces the capacity to incorporate hydrophobic compounds such as HNK (Almoustafa et al. 2017).

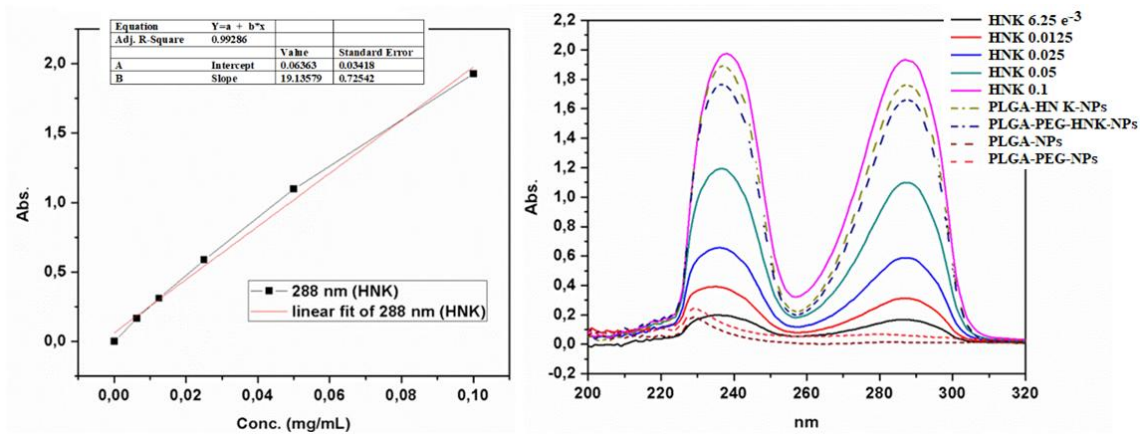


Fig. 6. HNK calibration curve and absorbance spectrum of HNK solutions at different concentrations and of all NPs produced

***In vitro* HNK cumulative release**

The release assays shown in Figure 7 were performed at two different pH values that simulate those of the stomach, intestine, and blood. After 2 h of contact between the NPs and the buffers used to simulate physiological conditions, more than 80% of HNK was released from the NPs, reaching about 100% after 15 h of the experiment. There were no major variations in the amount of HNK released as a function of pH. The rapid cumulative release of hinokinin from PLGA and PLGA-PEG nanoparticles, with more than 80% released within the first 2 h and nearly complete release after 15 h, is characteristic of burst release, a behavior commonly reported for PLGA-based systems (Lima et al. 2022a; Rezvantlab et al. 2018). The lack of significant pH dependence indicates that HNK release is mainly governed by diffusion from the polymeric matrix rather than polymer degradation under the tested conditions. This rapid release profile likely contributes to the lack of enhanced antiproliferative activity of HNK-loaded nanoparticles compared to free HNK in short-term (24 h) *in vitro* assays, highlighting the importance of release kinetics in interpreting biological performance (Rezvantlab et al. 2018).

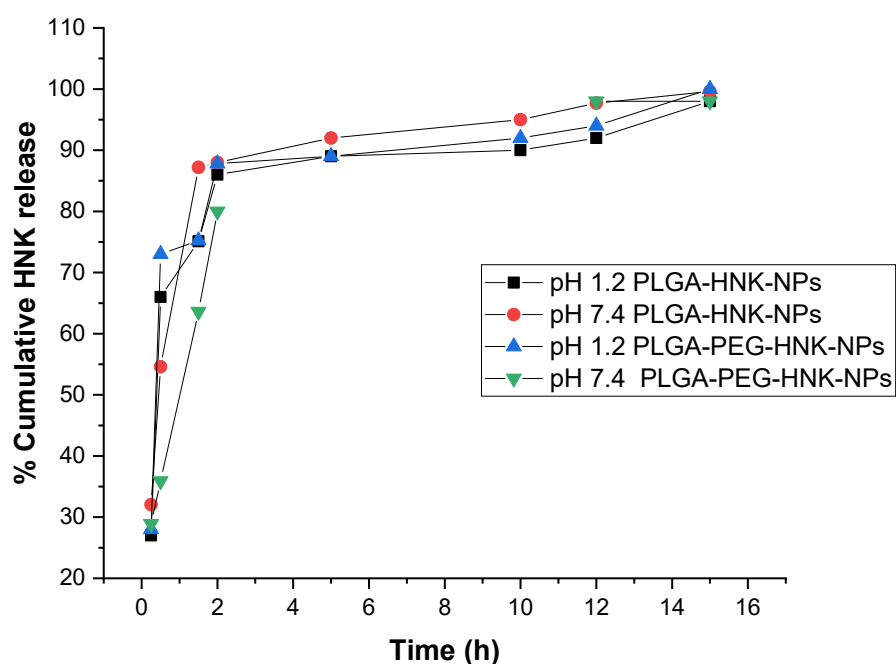


Fig. 7. *In vitro* release of HNK from PLGA-HNK-NPs and PLGA-PEG-HNK-NPs at pH 1.2 and 7.4

***In vitro* cytotoxicity of PLGA-HNK and PLGA-PEG-HNK NPs against tumor cell lines HCT116, A549, A2780**

In vitro assays were performed using the MTS colorimetric assay described in the experimental procedure after a 24h exposure of cancer cell lines to NPs. Three tumor cell lines were used in the cytotoxicity assays. All cytotoxicity assays were performed simultaneously, with doxorubicin, a common antitumor agent, used as a control (Figures 8, 9, and 10). Doxorubicin was used in all viability assays at the IC₅₀ concentration for HCT116 cell line (0.4 μ M) (Svahn et al. 2018).

For colorectal cancer HCT116 cells, a concentration-dependent reduction of cell viability was observed with HNK and HNK-loaded NPs (Figure 8). HNK alone induced an almost 40% reduction in cell viability at 75 μ M. NPs with HNK showed results similar to those with HNK, but with lower cell viability inhibition (20%). Unloaded control NPs (empty PLGA and PLGA-PEG) do not show a reduction of cell viability (for the same concentrations used in loaded NPs). Free HNK showed a higher inhibition of cell proliferation compared to loaded NPs, but lower than the positive control – doxorubicin, which reduced cell viability by more than 60% showed in Figure 8.

Analyzing the effect of free and NPs loaded HNK in the A2780 cell line, a similar trend to that of HCT116 was observed; however, a larger decrease in cell viability was

observed with the increased concentration of free HNK (Figure 9). Indeed, for HNK concentrations of 75 μM a reduction of cell viability of more than 40% was obtained (Figure 8). As previously, the HNK loaded NPs presented a lower reduction of viability compared to the free HNK. Interestingly, PLGA-PEG-HNK shows a better reduction of A2780 cell viability (almost 25%) compared with the unpegylated (PLGA-HNK) NPs (5% reduction) shown in Figure 10. It is interesting to note that PLGA-NPs show some reduction of viability (20%) compared to PLGA-PEG-NPs, which do not show any cytotoxicity, demonstrating in this case the biocompatibility of PEG in cells. Compared with the common antitumor drug Doxorubicin at 0.4 μM , a very high reduction in cell viability (>80%) was observed, indicating that this cell line is more sensitive to Doxorubicin than colorectal cancer cells (Figure 9).

The results presented in Figure 10 and the comparison of the effects of free HNK and HNK-loaded NPs in A549 cells, compared with the other two carcinoma cell lines, show that only free HNK reduces cell viability by 20% at 50 and 75 μM . PLGA-PEG-HNK shows a 10% reduction in cell viability at 75 μM . Unloaded NPs exhibit no cell toxicity (Figure 10). Interestingly, this cell line is more resistant to doxorubicin, and at the same concentration (0.4 μM), it shows only a 20% reduction in A549 cell viability.

Overall, the antiproliferative results indicate that hinokinin exerts a moderate, concentration-dependent effect on cancer cell viability that varies by tumor cell line and formulation. The consistently higher activity observed for free HNK compared to HNK-loaded nanoparticles suggests that the encapsulation strategy did not enhance short-term cytotoxicity under the experimental conditions employed. This behavior can be rationalized by the rapid release profile of HNK from PLGA-based nanoparticles, as previously demonstrated, which may limit the advantages of nanoparticle-mediated delivery during a 24 h exposure. In addition, differences among cell lines, particularly the reduced sensitivity of A549 cells to both HNK and doxorubicin, highlight intrinsic variations in drug responsiveness, membrane permeability, and cellular defense mechanisms.

The lack of cytotoxicity observed for unloaded PLGA and PLGA-PEG nanoparticles confirms the biocompatibility of the polymeric carriers, while the slightly improved activity of PLGA-PEG-HNK in A2780 cells suggests that PEGylation may influence cellular interactions in a cell-type-dependent manner. Compared with doxorubicin, which showed markedly greater inhibition of cell viability at submicromolar concentrations (Svahn et al. 2018), the results reinforce that HNK exhibits a distinct, less

potent antiproliferative profile, supporting its evaluation as a lead compound rather than as a direct alternative to conventional chemotherapeutics. Taken together, these findings emphasize the importance of integrating release kinetics, formulation properties, and cellular sensitivity to accurately interpret the biological performance of HNK-loaded nanoparticles.

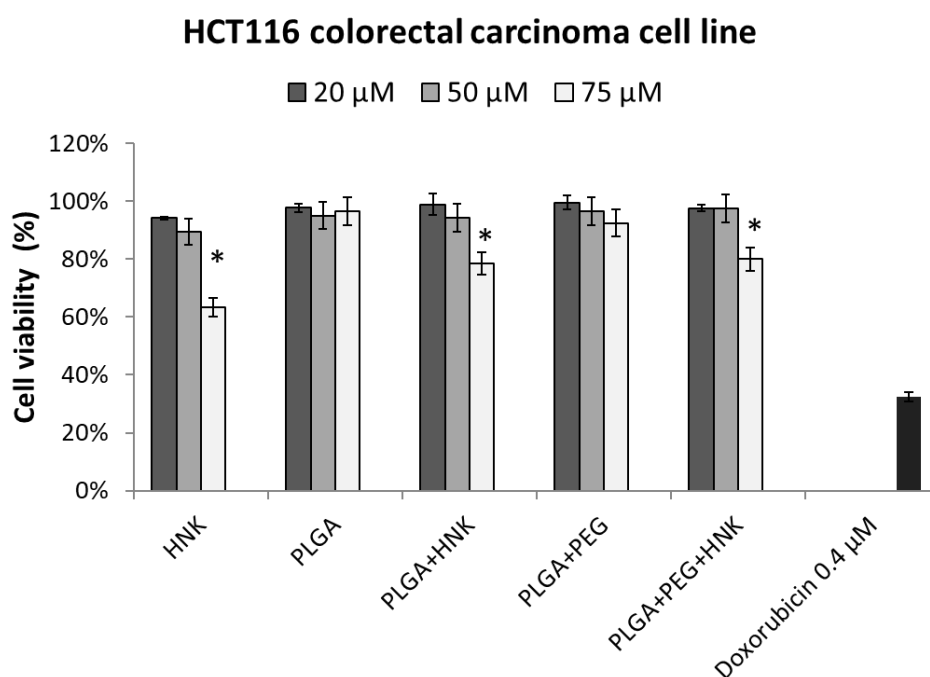


Fig. 8. Antiproliferative effect of HNK and PLGA NPs loaded and unloaded with HNK in human colorectal carcinoma cell line (HCT116). HCT116 cells were incubated for 24 h with increasing concentrations of HNK or NPs. Cell viability percentage was determined by comparison with HCT116 cells incubated with 0.1% DMSO (v/v) as the solvent control. The results shown are expressed as the average $\pm$ SD from three independent assays. The symbol * indicates that p-value < 0.05 (as compared to the control condition in Student's t-test)

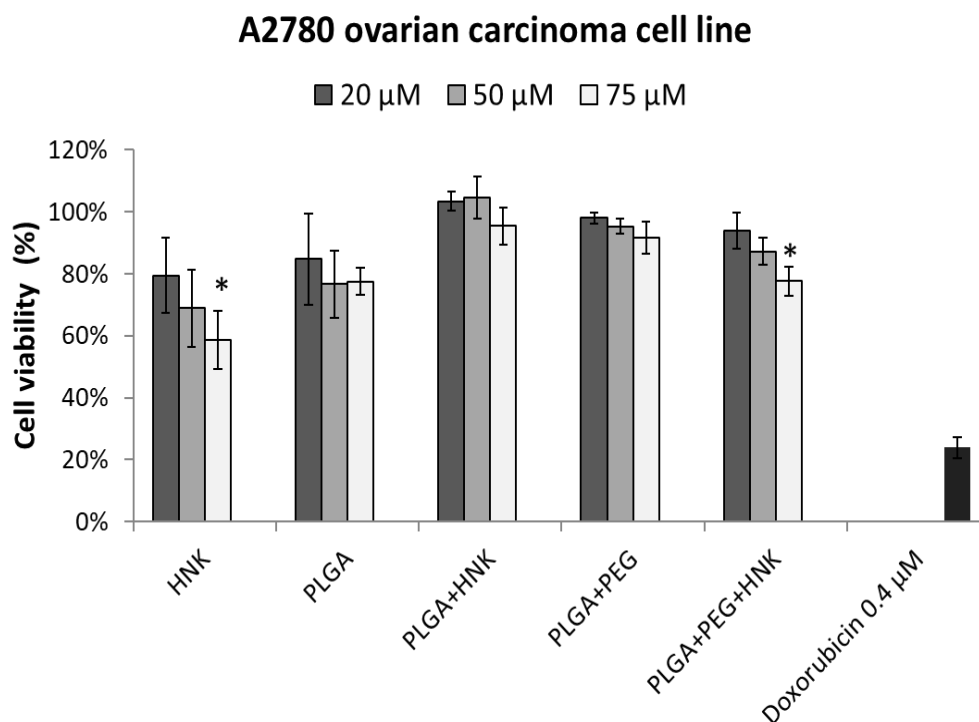


Fig. 9. Antiproliferative effect of HNK and PLGA NPs loaded and unloaded with HNK in the human ovarian carcinoma cell line (A2780). A2780 cells were incubated for 24 h with increasing concentrations of HNK or NPs. Cell viability percentage was determined by comparison with A2780 cells incubated with 0.1% DMSO (v/v) as the solvent control. The results shown are expressed as the average $\pm$ SD from three independent assays. The symbol * indicates that p-value < 0.05 (as compared to the control condition in the standard test)

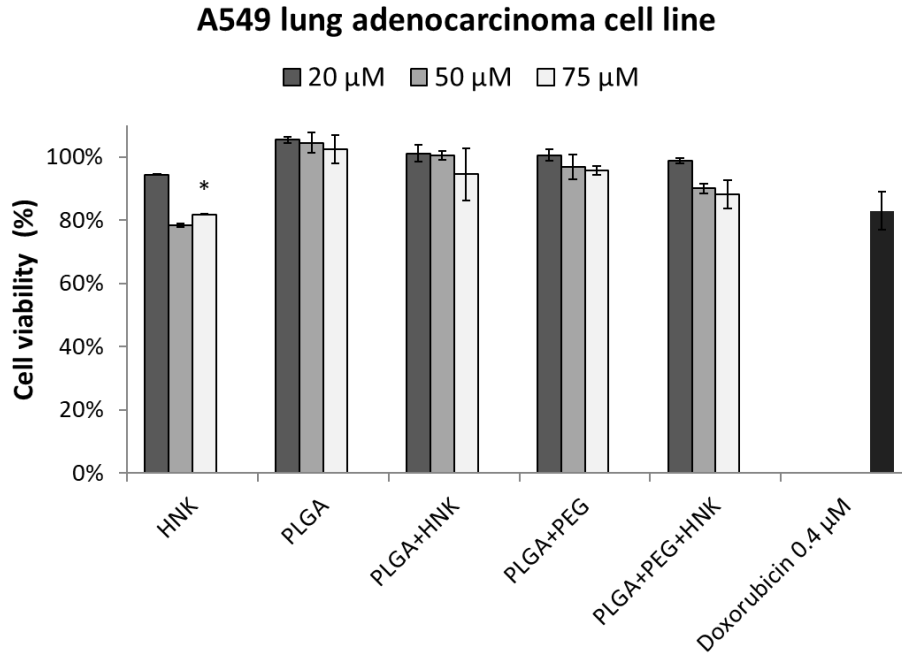


Fig. 10. Antiproliferative effect of HNK and PLGA NPs loaded and unloaded with HNK in human lung adenocarcinoma cell line (A549). A549 cells were incubated for 24 h with increasing concentrations of HNK or NPs. Cell viability percentage was determined by comparison with A549 cells incubated with 0.1% DMSO (v/v) as the solvent control. The results shown are expressed as the average $\pm$ SD from three independent assays. The symbol * indicates that p-value < 0.05 (as compared to the control condition in Student's t-test).

Integration of nanoparticle properties and antiproliferative activity

The physicochemical characterization confirmed the successful production of PLGA- and PLGA-PEG-based nanoparticles with sizes and surface charges comparable to those reported for similar polymeric systems (Lima et al. 2017a; Pissurno and Laurentiz 2017; Cao et al. 2013). High encapsulation efficiencies indicate favorable

Conclusions

Thus, hinokinin was successfully encapsulated in PLGA and PLGA-PEG nanoparticles with high encapsulation efficiency and suitable physicochemical characteristics, consistent with those of previously reported polymeric delivery systems. The rapid release profile observed may explain the lack of enhanced antiproliferative activity of HNK-loaded nanoparticles compared with free HNK in the tested cancer cell lines, whereas the unloaded nanoparticles demonstrated good biocompatibility. Taken together, these results highlight the potential of PLGA-based nanoparticles as delivery platforms for natural compounds with antiproliferative activity, and support further investigation of the molecular mechanisms underlying HNK cytotoxicity.

Authors' contributions

R.G. de Lima contributed to the investigation, formal analysis, and writing of the original draft of the manuscript. M.T. Barros and M.M. Cardoso contributed with resources and methodology. W.R. Cunha contributed with resources and conceptualization. E.G. Lucas and P.S.S.R. Cavallari participated in the investigation. P.V. Baptista and L.R. Raposo contributed with supervision and resources. A.R. Fernandes contributed to methodology and investigation. M.L. Andrade e Silva contributed to formal analysis and methodology. L. Menini contributed to investigation and writing – review and editing. L.A. Parreira contributed to methodology and investigation. M.F.C. Santos contributed to conceptualization, supervision, and writing – review and editing. R.S. Laurentiz contributed to conceptualization, supervision, funding acquisition, and writing – review and editing.

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Declarations

Competing interests

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.

Ethics approval and consent to participate

Not applicable. This study involved only in vitro experiments with established cancer-derived cell lines.

Consent for publication

Not applicable.

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