Abstract
Pharmaceutical nanotechnology is an emerging technology that proved its effectiveness in decreasing the side effects and improving the therapeutic outcomes of chemotherapeutic drugs. Combretastatin A4 (CA4) is a natural potent tubulin polymerization inhibitor. However, it suffers from low water solubility with various side effects. The aim of this study was to formulate and characterize poly(lactide-co-glycolide) (PLGA) nanoparticles loaded with CA4, to assess its release kinetics and to evaluate the in vitro cytotoxic activity of the obtained nanoparticles. CA4 was synthesized according to a previous protocol. Nano-precipitation and emulsion evaporation methods were used to produce the desired drug-loaded PLGA nanoparticles. The obtained nanoparticles were characterized for shape, particle size, zeta-potential and morphology. PLGA nanoparticles produced by the emulsion evaporation method exhibited higher EE and DL than those prepared by the nanoprecipitation method and hence were selected for further release and cytotoxicity studies. The nanoparticles showed sustained release pattern, following zero-order kinetics. In vitro cytotoxicity studies demonstrated the superiority of CA4 loaded nanoparticles over the corresponding free CA4. CA4 loaded nanoparticles were successfully produced and showed satisfactory characteristics. In addition, an improvement in the cytotoxic and IC50 of CA4 loaded NPs was demonstrated. This suggests that these nanoparticles could be used to improve the safety, effectiveness, and patient compliance, which will be further investigated by in vivo studies.

1. Introduction
Nowadays polymeric nanoparticles are frequently used for various applications in the pharmaceutical field [1]. For instance, biodegradable and biocompatible poly (lactide-co-glycolide) (PLGA) nanoparticles are used to improve the efficacy and safety of various water-soluble and insoluble drugs [1–5]. These pharmaceutical products have several advantages including (i) inhibition of a premature degradation of the active pharmaceutical ingredient (API), (ii) decreasing the interaction of the API with the biological environment, (iii) enhancement of absorption of the API into a targeted tissue, (iv) enhancement of its bioavailability, retention time and improvement of the intracellular penetration of the API [6]. The most common methods used to formulate these nanoparticles are (i) emulsion-evaporation, (ii) nano-precipitation, (iii) dialysis, (iv) salting out, (v) emulsion diffusion, (vi) and spray drying [7–10]. Therefore, the selected method and used polymer are usually based on the physicochemical properties of the drug [8]. In addition, the final properties of the obtained nanoparticles (size, zeta-potential and morphology) will depend on the method of preparation, concentration,
and composition of the used polymer, the viscosity of the system, stabilizer, and type of the organic solvent involved [8, 9]. The emulsion evaporation method is a technique in which an organic layer, composed of the polymer and a suitable organic solvent (water-immiscible organic solvent like dichloromethane) is emulsified in an aqueous layer containing a suitable stabilizer. After that, the organic solvent is evaporated to produce a suspension of nanoparticles [8, 11, 12]. In the nanoprecipitation method, the drug and polymer are dissolved in a water-miscible organic solvent which is added to the aqueous phase to produce nanoparticles. After that, the organic layer is removed by stirring at room temperature or by reducing the pressure [8, 11, 12]. This easy and fast technique usually produces nanoparticles with a narrow size distribution, duplicate scale-up method, no energy for homogenization and no need for a large amount of toxic organic solvents [12].

Combretastatin A4 (CA4) is a natural anticancer agent that is isolated from willow Combretum caffrum (Eckl. and Zeyh.) Kuntze [13–15]. It belongs to a group of drugs known as vascular disrupting agents (VDA) that bind to colchicine binding site in microtubules to inhibit their polymerization. In addition, it has anti-vascular effects at a dose below the maximum tolerated dose which improves its safety profile [13, 16, 17]. Moreover, CA4 inhibits angiogenesis via inhibition of vascular endothelial growth factor (VEGF) and inhibits subsequent activation of vascular endothelial growth factor receptor-2, a receptor that mediates angiogenesis [18]. In addition, CA4 causes a decrease in mitochondrial oxygen consumption and thus affect the ability of the tumor cells to angiogenesis [19]. Another mechanism for CA4 suggested a cleavage and activation of caspase 3 and 9 proteins, which upon activation lead to apoptosis and cell death [16]. From a physicochemical point of view, CA4 is a water-insoluble and chemically unstable compound. In fact, it suffers from de-activation during storage due to a spontaneous transformation of cis-isomer into the inactive trans-isomer by light or heat. Accordingly, many derivatives like CA4 triphosphate (CA4P) and cis-locked chemical structures were synthesized with the aim to increase the water solubility and chemical stability of this agent [15, 16, 20–24].

On the other side, the conversion of CA4 into its phosphate prodrug would increase the synthetic steps which increase the final cost of the drug. In addition, this water-soluble prodrug would increase the side effect and decrease the efficacy of CA4, if used as an injectable solution, since it may result in high vascularization which causes a lack of targeting the tumor cells only. Accordingly, using the CA4 form in suitable polymeric nanoparticles would avoid the aforementioned inconveniences.

CA4P is in phase II/III clinical trials approved for treating thyroid malignancy by Oxigene Company [20, 25, 26]. Chemically, CA4 has two aromatic rings which are connected to each other by ethylene bridge in cis isomer (figure 1).

In the last decade, many attempts were conducted to produce CA4 nanoparticles in order to reduce its toxicity and improve its therapeutic effectiveness. In 2010, Yiguang Wang et al developed a tumor vasculature-targeted polymeric micelle containing CA4 using poly(ethylene glycol)-b-poly(d,l-lactide) copolymer. The study showed superior efficacy of the obtained CA4 micelles over nontargeted micelles [27]. Nanoparticles loaded with both CA4 and paclitaxel to treat tumor vascular endothelial cells using, arginine-glycin-aspartic acid (RGD) was developed [28]. Efficient uptake of both drugs by human umbilical vein endothelial cells were observed and an improvement in both potency and cytotoxicity of these drugs was recorded [28]. In 2017, micelle-like CA4 nanoparticles were developed to release CA4 in the acidic tumor cells which might result in an increase in the circulation time and CA4 stability causing an improvement in the therapeutic outcome of CA4 [25]. In 2018, a study was conducted in our lab to load CA4 into functionalized carbon nanotubes to produce an SWCNT-CA4 system in order to increase the solubility of CA4 and targeting toward cancer cells. The study demonstrated that SWCNT-CA4 had significant necrotic effects on HeLa cells, making it superior as a therapeutic agent than free CA4 in the treatment of cancer [29]. As the ester linkage between the CA4 and the SWCNTs could be hydrolyzed in the blood esterase enzyme, this would release free CA4 to the blood circulation and may cause side effects. Therefore, in this paper, we aim to encapsulate the CA4 into PLGA polymeric NPs that would avoid this inconvenient. In fact, to the best of our knowledge, this encapsulation strategy of the CA4 has not been reported elsewhere.
Accordingly, in this study, we aim to encapsulate the CA4 into PLGA nanoparticles to obtain a long sustained release system that would protect the CA4 from the blood circulating enzymes and increase the CA4 targeting toward tumor which will decrease the side effects of CA4, and increase the half-life of the drug that decrease the need for multiple dosing so increasing the patent compliance.

2. Materials and methods

2.1. Materials

All materials that were used in synthesis CA4 and PLGA nanoparticles were of analytical grades. 2-(3,4,5-trimethoxyphenyl) acetic acid, 3-hydroxy-4-methoxybenzaldehyde, and quinoline were purchased from Alfa Aesar Company (England). Acetic anhydride and copper were purchased from Sigma-Aldrich (USA). Hexane and ethyl acetate were obtained from Frutarom. In order to purify the obtained reaction products, column chromatography using silica gel (pore size 60 Å, 40–63 μm particle size, 230–400 mesh particle size) was used. This silica was purchased from Sigma-Aldrich Company (USA). The reactions were monitored using TLC (DC-Fertigfolien Alugeram® Silg/Uv254, Macherey Nagel Company (Germany). PLGA (Resomer® RG 502H, Evonik, Germany) with Lactide to glycolide ratio of 50:50 and MW of 7–17 kDa was used for the preparation of nanoparticles. Acetone from Fisher chemical (UK), dichloromethane from AZ Chem company (China), polyvinyl alcohol (PVA) 99% hydrolyzed powder with MW 89–98 kDa from Sigma-Aldrich (USA), methanol from Romil pure chemistry, acetonitrile from Scharlab (Spain). Dialysis tubing cellulose membrane, with flat width 43 mm and MWCO 14 kDa, was used to conduct the in vitro release study. This tube was purchased from Sigma-Aldrich Company (USA). Materials that are used in the preparation of buffer solution are sodium dihydrogen phosphate anhydrous from AZ Chem for chemical (India), potassium dihydrogen phosphate from AZ Chem for chemicals (China), sodium phosphate dibasic dihydrate from Sigma-Aldrich (Germany), potassium chloride from Xiong chemical industry (China), sodium chloride from AZ Chem for chemical (Germany). For tissue culture, Dulbecco’s Modified Eagle Medium (DMEM) growth culture media and penicillin-streptomycin solution 100X were purchased from Euro-clone, Italy. Phosphate buffer saline 1X from Eurobio, France. Trypsin ethylenediaminetetraacetic acid (EDTA) 10X solution from Santa Ana, California, and dimethyl sulfoxide (DMSO) from Scharlab, Spain, while Thiazolyl Blue Tetrazolium Bromide from Sigma-Aldrich, USA. All the materials that were used for in vitro cytotoxicity assay are for cell tissue culture use grade.

2.2. Methods

2.2.1. Synthesis and characterization of CA4

CA4 was synthesized according to the method published by Xiao et al with some modification as reported by Assali et al [29, 30]. NMR analysis was used to confirm the structure of the synthesized CA4 according to our previous work. Precisely, 4.33 ml (46.41 mmol) of acetic anhydride and 2.2 ml (15.91 mmol) of triethylamine were added to 3 g (13.26 mmol) of 3,4,5-trimethoxyphenyl acetic acid and 2.421 g (15.9 mmol) of 3-hydroxy-4-methoxy benzaldehyde. The reaction was carried out using 50 ml of DCM and heated to 110-degree centigrade under reflux for 4 h. The reaction was cooled to room temperature (RT) and then was acidified using 2 M HCl and kept overnight in an ice bath. The day after, a dark brown precipitate was found. The supernatant was discarded and 30 ml of 10% NaOH solution was added to dissolve the brown precipitate and washed using 100 ml ethyl acetate. After that, the cinnamic acid derivative was precipitated using 2 M HCl. The product was filtrated and dried. Then the dried product was recrystallized using ethyl acetate.

4.66 g of copper and 3.3 g of cinnamic acid derivative were added to 15 ml quinoline under argon conditions and magnetic stirring for 3 h at 200 °C. After that, the reaction was cooled to the RT and the copper was filtered away and CA4 was extracted using 100 ml ethyl acetate and washed using a 50 ml 2 M HCl solution, then the organic layer was cleaned using 50 ml of saturated NaCl solution. The cleaned organic phase was dried using anhydrous Na2SO4 to obtain a viscous brown fluid that contains CA4 product. CA4 was purified using flash column chromatography with a mobile phase composed of hexane: ethyl acetate (7:3). Further purification by recrystallization was carried out by ethyl acetate: petroleum ether (1:9) to obtain white powder CA4.

2.2.2. Preparation of CA4 loaded PLGA nanoparticles

Several trials were conducted to prepare PLGA NPs, using both nanoprecipitation and emulsion evaporation methods.

2.2.2.1. Formulation by nanoprecipitation method

In this method [10], 50 mg or 100 mg of PLGA 502 H and 1 mg of CA4 were dissolved in 2 ml acetone and added dropwise into 5 ml phosphate buffer (pH = 7.4) under magnetic stirring (digital multi stirrer, model number F203A0179, Italy) at 400 RPM and left overnight until complete evaporation of acetone. The CA4 loaded PLGA...
NPs were collected by centrifugation (Hermle, model number Z216M, Germany) at relative centrifugal force (RCF) of 21379 x g for 10 min. The supernatant (containing free drug) was discarded while the sediment particles were washed with methanol to remove the free unloaded drug. NPs were collected by centrifugation as mentioned above. Blank PLGA NPs were prepared similarly but without the using CA4 drug.

### 2.2.2.2. Formulation by the emulsion evaporation method

In this method [8], several trials were conducted with different amounts of CA4 and PLGA 502 H (table 3). Known amounts of PLGA and CA4 according to the formula were dissolved in 1 ml dichloromethane (DCM) and emulsified with 5 ml of aqueous phase containing 1% PVA using ultra-probe sonicator (Hielscher, model number UP200Ht, Germany) at an energy output of 50 W for 30 s over an ice bath. Then, the O/W emulsion was kept under magnetic stirring (digital multi stirrer, model number F203A0179, Italy) at 400 RPM overnight until complete evaporation of DCM. The CA4 loaded PLGA NPs were collected by centrifugation (Hermle, model number Z216M, Germany) and the NPs were washed with methanol, centrifuged and collected for further characterization. Blank PLGA NPs were prepared similarly as CA4-loaded PLGA NPs but without using CA4.

### 2.2.3. Characterization of CA4 loaded PLGA NPs

#### 2.2.3.1. Particle size and zeta-potential

Particle size and zeta-potential values of both blank and CA4 loaded PLGA NPs were measured using Zetasizer Nano-ZS Malvern (Model number ZEN3600, UK).

#### 2.2.3.2. Encapsulation efficiency and drug loading

Encapsulation efficiency (EE%) and drug loading (DL%) of CA4 loaded PLGA NPs were calculated by dissolving determined quantities of these NPs in 2 ml acetonitrile (ACN) and left stirring for 3 h to ensure complete solvation of both PLGA polymer and CA4 drug. The resulting solution was then measured using a UV–vis spectrophotometer at 300 nm. EE% and DL% were calculated according to equations (1) and (2).

\[
\%EE = \frac{\text{Amount of loaded CA4(µg)}}{\text{Amount of initial weight of CA4(µg)}} \times 100
\]  

\[
DL\% = \frac{\text{Amount of loaded CA4(µg)}}{\text{Weight of CA4 loaded PLGA NPs(µg)}} \times 100
\]

A CA4 Calibration curve was constructed in order to calculate the amount of CA4 loaded in PLGA NPs. For this purpose, 50 µg 1 ml⁻¹ concentration of CA4 in acetonitrile was prepared as a stock solution (\(\lambda_{\text{max}} = 300\) nm). After that, series concentrations of CA4 (40, 30, 21, 10.5, and 5.25 µg ml⁻¹) were prepared by serial dilution from the stock solution using acetonitrile. Then, the concentration of CA4 in the solution was measured by UV–vis spectrophotometer (Evolution 220, 840–210600, USA) at 300 nm as shown in figure S2.

#### 2.2.3.3. Morphology of CA4 loaded PLGA NPs and blank NPs

The morphology of both blank and CA4 loaded PLGA NPs was examined by the transition electron microscope (TEM) (Morgagni 286 transmission microscope (FEI Company, Eindhoven, Netherlands) at 60 kV). For this purpose, one drop of nanoparticle suspension was added to the carbon-coated copper grid.

#### 2.2.4. Mathematical calculation

The 2² factorial design was constructed to investigate the impact of the concentration PLGA and CA4 on the particle size and EE% of the obtained NPs. The two independent variables are PLGA/CA4 ratio as (X1) and CA4 as (X2). Each independent variable had 2 levels which were coded as –1 and +1. The coded values of independent variables were given to the software (Appendix 1). The four runs in the design matrix of 2² full factorial designs are set up by randomization. A multiple regression, first-degree model was used to express the response as a function of the selected factors (Appendix 1). The response variables measured are the z-potential, EE% and particle size. Design-Expert version 8.0.7.1 was applied for performing the experimental design and the data analysis [31]. A multiple regression, first-degree model was used to express the response as a function of the selected three factors (Appendix 1). A multiple regression, first-degree model was used to express the response as a function of the main effects. ANOVA Test (\(p < 0.05\)) statistically confirmed the significance of effects of variables and their interaction.
2.2.5. Release of CA4 from PLGA nanoparticles
For release study, 85 mg of CA4 loaded PLGA nanoparticles were suspended in 2 ml PBS (pH = 7.4) containing 1% w/v SLS and placed into a dialysis bag. The dialysis bag was immersed in 14 ml of the same above mentioned media and kept shaking at 100 RPM and 37 °C using shaker incubator (Lab Tech, model number LSI-3016A, Korea). Samples (1 ml each) were withdrawn from the release media at specific time points and replaced with the same amount of fresh media to maintain the sink condition. The amount of CA4 released was measured using a UV–vis spectrometer at 300 nm. The same procedure was carried out for the blank NPs. Measurements were performed in triplicate. The release data were analyzed to describe the release behavior of the CA4 from PLGA NPs using several kinetic release models such as zero-order, first-order, and Higuchi (appendix 2).

2.2.6. In vitro cytotoxicity
The anticancer activity of free CA4, blank NPs and CA4 loaded PLGA NPs was assessed against Caco-2 cancer cell lines.

2.2.6.1. Cell culture
Caco-2 cells were grown using 15–cm² plastic culture plates. The culture growth medium (CGM) was composed of DMEM medium. 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were used as a supplement for the medium. Cells were incubated in the above prepared medium and kept at 37 °C in a humidified atmosphere enriched with 5% CO₂. For sub-culturing, the obtained CGM was suctioned from the culture plates. After that, the cells were washed using 7 ml of Ca²⁺–free PBS. Then, 1 ml of trypsin was added to the cells and were incubated for 5 min under the above-stated growth conditions. After that, 3 ml of CGM was added under gentle mixing to inactivate the trypsin, and subsequently, the cell suspension was placed into 96-well plate in an appropriate cell number and left for 24 h to allow appropriate adherence of the cells to the plates.

2.2.6.2. Cell viability test
Different concentrations of free CA4 and drug-loaded NPs were added to cancer Caco-2 cell lines and incubated under the above-mentioned conditions. Reading of the % viable cells were recorded at 24 and 48 h. Then, 20 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solutions were added to each well followed by an incubation period of 3 h, then the media were removed and 200 μl of DMSO was added to dissolve the crystal and the absorbance was recorded at 530 nm wavelength using plate reader (BioTek, USA). GraphPad Prism 7 was used to estimate the IC₅₀ values by fitting curves (log concentration vs. normalized cell viability %) with a nonlinear regression model using dose-response-inhibition equation.

3. Results

3.1. Synthesis and characterization of CA4
In this study, CA4 was successfully synthesized and purified giving a white powder with a melting point of 116 °C and yield around 36.4% [32]. ¹H NMR spectrum was also conducted to prove its identity the synthesized product (figure S1 is available online at stacks.iop.org/MRX/6/1250d7/mmedia). According to ¹H NMR (300 MHz, CDCl₃): δ 6.90 (s, ¹H, CH, C₁₁ Ar), 6.77 (d, J = 8.19 Hz, ¹H, CH, C₁₁ Ar), 6.67 (d, J = 8.19 Hz, ¹H, 1CH, C₁₁ Ar), 6.51 (s, 2H, 2CH, Ar), 6.39 (d, J = 12.1 Hz, ¹H, CH=CH), 6.45 (d, J = 12.1 Hz, ¹H, CH, CH=CH), 5.50 (s, ¹H, OH), 3.82–3.84 (s, 6H, 2OCH₃), 3.68 (s, 6H, 2OCH₃).

After the successful synthesis and purification of CA4, many attempts were conducted to encapsulate it within PLGA NPs. For this purpose, two formulation techniques, emulsion evaporation, and nanoprecipitation were tried with an attempt to find the best one in terms of EE%, shape, size and release profile. In addition, we benefit from the biodegradable characteristic of PLGA polymer in synthesizing anticancer CA4 loaded PLGA NPs as a controlled drug delivery system [33]. Herein, several parameters were controlled and varied in order to obtain the best condition for the optimum formulation with the highest EE% and small particle size. These parameters included the method of preparation of NPs, amount and type of the used polymer, and the amount of CA4 used (tables 1 and 2).

3.1.1. Effect of the used method

3.1.1.1. Nanoprecipitation method
Suitable small blank and CA4 loaded NPs were successfully produced by the nanoprecipitation method. As can be shown in table 1, increasing the amount of PLGA resulted in an increase in particle size. For example, increasing PLGA amount for blank nanoparticles from 50 to 100 mg resulted in an increase of particle size from 118.5 to 192.9 nm. A similar trend was observed with drug-loaded counterparts. The increase in the amount of
PLGA in the organic phase results in a higher amount of PLGA in aceton droplets leading to larger sizes upon evaporation of the organic solvent. This results in an increase in the droplet size of the organic phase viscosity of the emulsion, so the sonicator becomes less effective in breaking the particles into smaller ones. In fact, this hypothesis was also confirmed by other studies [32, 33].

Regarding the surface charge, both blank and loaded PLGA nanoparticles exhibited negative zeta-potential values in the range of $-69$ to $-78$ mV as shown in table 1. In fact, the used PLGA encompasses a carboxylic ending group which results in the anionic surface charge. Regarding drug loading and EE%, It can be seen that formulations prepared with 50 mg of PLGA and 1 mg of CA4 (Formula A3) resulted in drug loading of 0.6% and EE% of 31%. On the other hand, doubling the amount of polymer resulted in a similar EE% of 32% with a lower drug loading of 0.4%. This would be not straightforward to explain but would be due to the limited capacity of the encapsulating particles. Concerning the surface morphology of the obtained NPs, both drug-loaded and blank PLGA NPs were assessed using TEM. The shape of the particles that were measured revealed that they have almost spherical in shape with a relatively smooth surface with minor fluctuations in the obtained sizes (figure 2). Moreover, a representative TEM image was used to develop a histogram distribution of particle size by applying Sturges rule ($k = 1 + 3.322 \log N$ where $N = 71$). The obtained histogram is fitting the log-normal model with a mean particle size (±SD) of $184 \pm 18$ nm as shown in figure 2(C) [34].

### 3.1.1.2. Emulsion evaporation method

It was able to achieve higher EE% than the nano-precipitation method. Accordingly, this method was adopted as a master preparation method to produce NPs. However, additional trials were conducted to improve the quality of the obtained NPs such as EE%, zeta-potential, and particle size (table 2).

As shown in table 2, PLGA nanoparticles with varying amounts of PLGA (50 or 100 mg) and CA4 (0–8 mg) were prepared by the emulsion evaporation method. All batches exhibited similar particle sizes in the range of (182 to 208 nm) with no significant effect of varying the amount of PLGA in contrast to that shown with nanoparticles prepared with the nanoprecipitation method (table 1). The reduced effect of the polymer amount on particle size would be attributed here to the sonication effect which results in similar sizes regardless of the different viscosities produced with varying amounts of PLGA. As also shown in table 2, zeta potential values in the range of ($-25$ to $-39$ mV). The lower zeta potential values observed with these nanoparticles than those prepared by the nanoprecipitation method is attributed to the adsorption of PVA polymers at the surface of these particles. Regarding drug loading and EE%, It can be seen that formulations prepared with 50 mg of PLGA and 1 mg of CA4 (Formula A7) resulted in drug loading of 1% and EE% of 40.92%. On the other hand, doubling the amount of the drug while keeping the same amount of PLGA (Formula A8) resulted in higher drug loading (1.6%) but with significantly lower encapsulation efficiency (29%). The lower encapsulation efficiency is attributed to the limited capacity of PLGA nanoparticles (i.e. the same amount and size) leading to wasting the drug as surplus. The effect of increasing the amount of PLGA on drug loading and EE% was also studied. A formula with PLGA amount of 100 mg and 1 mg of CA4 exhibited drug loading of 0.5% and EE% of 46%. Increasing the amount of drug to 2, 4 and 8 mg resulted in higher drug loading values of 1, 2 and 3%, respectively. Such an increase in drug loading was accompanied by a general decrease in the EE% (from 51% to 36%) probably due to the limited capacity of PLGA as mentioned above.

### 3.1.1.2.1. Statistical assessment

For this purpose, $2^3$ factorial design was adopted to assess the statistical impact of the concentrations of PLGA/CA4 ratio and the amount of added CA4 on EE%, zeta-potential and particle size of the obtained NPs (table 3). Several statistical models were used to find the most significant one. Unfortunately, all suggested models were
Table 2. Formulation and observed quality of NP by the emulsion evaporation method.

| Components       | A5     | A6     | A7     | A8     | A9     | A10    | A11    | A12    |
|------------------|--------|--------|--------|--------|--------|--------|--------|--------|
| PLGA (mg)        | 50     | 100    | 50     | 50     | 100    | 100    | 100    | 100    |
| CA4 (mg)         | —      | —      | 1      | 2      | 1      | 2      | 4      | 8      |
| PVA (mg)         | 50     | 50     | 50     | 50     | 50     | 50     | 50     | 50     |
| DCM (ml)         | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      |
| Water (ml)       | 5      | 5      | 5      | 5      | 5      | 5      | 5      | 5      |
| Particle size (nm) ± SD | 196 ± 4 | 208 ± 0 | 193 ± 2 | 182 ± 2 | 207 ± 4 | 203 ± 1 | 205 ± 3 | 200 ± 0 |
| PDI ± SD         | 0.14 ± 0.01 | 0.14 ± 0.01 | 0.12 ± 0.02 | 0.10 ± 0.01 | 0.14 ± 0.01 | 0.20 ± 0.03 | 0.15 ± 0.02 | 0.12 ± 0.02 |
| Z-potential (mV) ± SD | −28 ± 1 | −37 ± 0 | −25 ± 0 | −33 ± 1 | −37 ± 1 | −39 ± 1 | −34 ± 2 | −34 ± 0 |
| EE% ± SD         | —      | —      | 41% ± 3 | 29% ± 2 | 46% ± 0 | 51% ± 6 | 42% ± 1 | 36% ± 4 |
| DL% ± SD         | —      | —      | 0.93% ± 0.06 | 1.60% ± 1.40 | 0.51% ± 0.04 | 1.13% ± 0.09 | 1.86% ± 0.17 | 3.43% ± 0.46 |
not significant. We adopted the none model which showed the highest F value and the lowest p-value, except for EE% where the root square method was significant (F value = 410 and the p-value was less than 0.05). In this context, the results of this factorial design showed the positive but not significant effect of the ratio of PLGA/CA4 on EE% (p-value was higher than 0.05). Vice versa, the concentration of CA4 had a positive and significant effect on both particle size and EE% (p-value less than 0.05).

Concerning zeta-potential, both PLGA/CA4 ratio and CA4 had a negative but not significant effect as shown in half-normal plot (Appendix 3).

Accordingly, formula A10 was adopted to produce the desired CA4 loaded PLGA NPs. In fact, perform optimization was not conducted since all used models were not significant. Accordingly, further studies should take into consideration other variables or other formulation methods to optimize our responses.

### 3.1.2. In-vitro release of CA4 from PLGA NPs

The time-dependent release of CA4 from PLGA NPs (Formula A10) in PBS pH = 7.4 containing 1% SLS 1% at 37 °C was studied. PLGA NPs exhibited sustained release behavior of CA4 with around 87% released over 17 days as shown in figure 3.

CA4 loaded PLGA NPs showed an interesting long sustained release pattern of about 17 days to release most of the encapsulated CA4. In fact, this release behavior, accompanied by the reasonable particle size (around 200 nm), would permit the safe circulation of the NPs in the bloodstream without releasing a toxic dose of CA4 in the healthy tissue, but at the same time accumulates into the tumor tissue. Kinetic analysis was performed to understand the release pattern of CA4 from the obtained PLGA NPs. As shown in table 4, release data were fitted...
against zero order, first order and Higuchi model and the linear regression ($R^2$) values were calculated. The results suggest that drug release follows zero-order kinetics as indicated with the $R^2$ value of 0.9875 [35, 36–40] (table 4).

### 3.2. Anticancer activity

The MTT cell viability assay was used to investigate the cytotoxicity of free CA4 and CA4 loaded NPs against Caco-2 cells as shown in figure 4. In addition, 9 different concentrations of the drug were used in order to calculate the IC$_{50}$.

As shown in figure 4, the calculated IC$_{50}$ values for the free drug was 67.9 ng ml$^{-1}$ while that for drug-loaded NPs of around 25.1 ng ml$^{-1}$. It is worth noticing that the results of in vitro cytotoxicity showed a time-dependent activity of both free and loaded drugs. Precisely, no significant effect was obtained after 24 h of treatment for the free drug or the charged NPs. However, both products showed marked activity after 48 h of incubation.

In fact, the blank NPs showed no cytotoxic effect on the cultured cell lines. This suggests that producing CA4 loaded PLGA NPs would improve the anticancer effect of CA4. In addition, the obtained NPs would cause fewer side effects due to the targeting and release patterns of these NPs.

### 4. Conclusions

The emulsion evaporation method was successfully used to produce CA4 loaded PLGA NPs with satisfactory EE%. The obtained NPs were almost spherical in shape with a relatively smooth surface. The in vitro release showed an interesting zero-order sustained release pattern over 17 days. This would permit the safe circulation of the NPs in the bloodstream without releasing a toxic dose of CA4 in the healthy tissue, but at the same time accumulates into the tumor tissue. Furthermore, these NPs were showed more significant cytotoxic effect against Caco-2 cell lines, when compared with the free CA4. These results suggest that formulating CA4 in PLGA NPs would be of great importance in order to improve the safety and efficacy of this interesting cytotoxic drug. However, in vivo studies using animal models should be conducted in order to confirm these results.

### Funding

SRA and AA acknowledge the financial support of the Deanship of Scientific Research at the University of Jordan. ANZ and MH acknowledge the financial support of the Faculty of Graduate Studies at An-Najah National University.
Conflicts of interest

The authors declare no conflict of interest

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