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Pomegranate extract-loaded surfactant-free zein nanoparticles as a promising green approach for hepatic cancer: optimization and in vitro cytotoxicity

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Abstract

Background Hepatic cancer endures a major health scourge as the consequence of a high incidence of > 1 million cases by 2025. Plant-based products are typically effective in ameliorating health conditions. Pomegranate peel extract (PE) with its high polyphenolic content has anticancer effects against different types of cancer. Herein, we aimed to maximize the PE chemotherapeutic efficacy by loading it in a suitable delivery system to overcome the limitations of PE, to control its release and to achieve liver targeting.

Method A nanoprecipitation procedure was adopted to incorporate PE into biodegradable and biocompatible natural polymeric zein (ZN)-based nanoparticles (NPs) (PE-ZN NPs). A full factorial design ($2^2 \times 3^1$) was developed to study the effects of the formulation variables, namely pH of dispersion, PE-to-ZN ratio and surfactant concentration.

Results The optimization revealed a surfactant-free stable PE-ZN NPs formula with a small particle size of 99.5 ± 6.43 nm, high PE encapsulation efficiency % of 99.31% ± 3.64 (w/w) and controlled release of PE over 24 h.

Conclusion Moreover, the cytotoxicity of the optimum formula against hepatic cancer HepG2 cell lines was assessed and attained about a 2.5-fold reduction in the inhibitory concentration (IC_{50}) values compared to the free PE affording a promising green platform to combat hepatic cancer.

Keywords Pomegranate peel extract, Zein, Polymeric nanoparticles, Anticancer, HepG2

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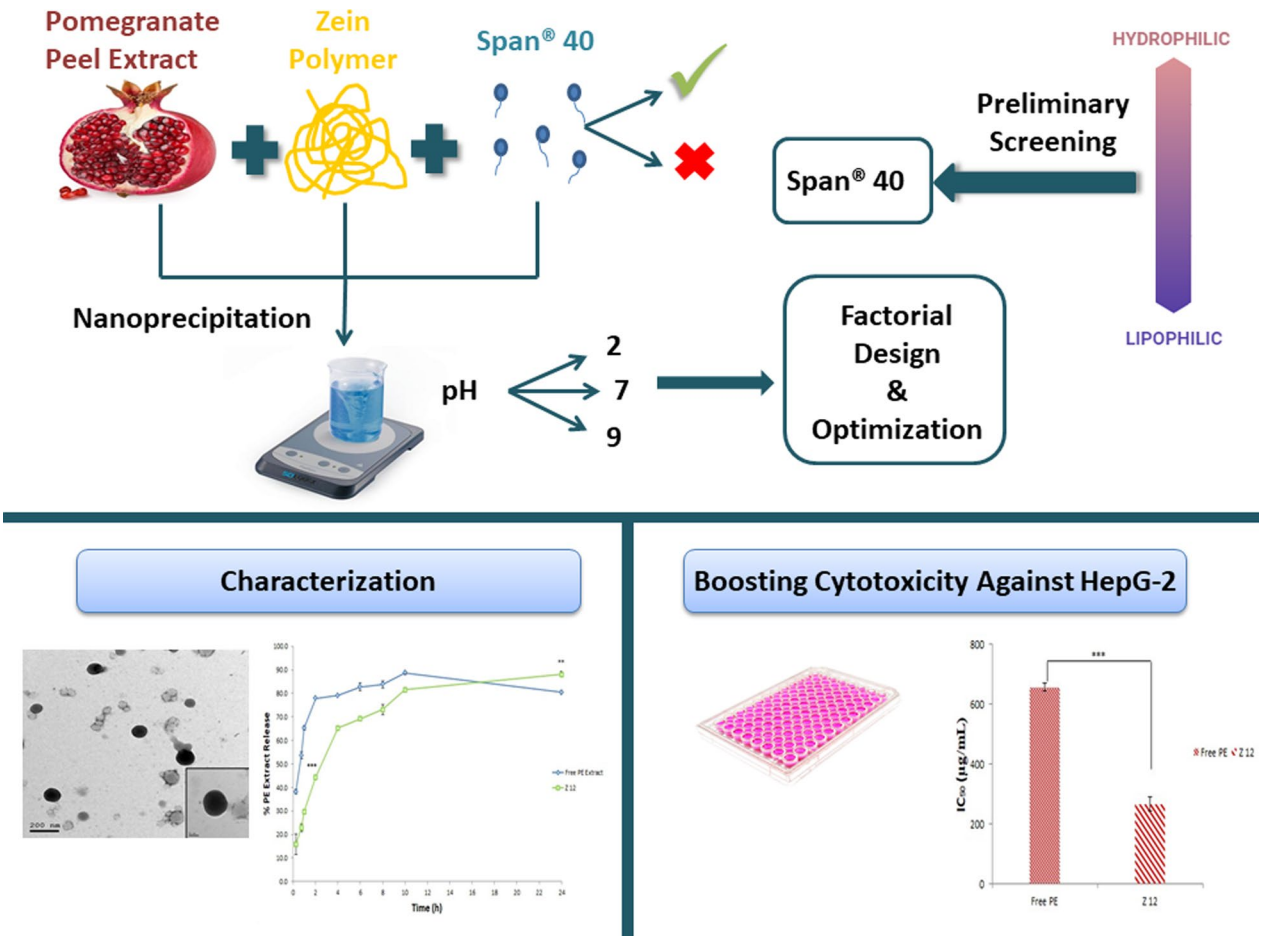
Graphical abstract



Background

Cancer is considered one of the main causes of mortality worldwide as a consequence of millions of new reported cases yearly. Hepatic cancer is the sixth most common cancer globally, ranking the fifth in men and the ninth most commonly occurring cancer in women [16]. The prognosis for hepatic cancer is poor, and there are many challenges for its cure. Liver resection and liver transplantation are the best options of cure for only 5–15% of early-stage patients; however, the high postoperative recurrence and the severe shortage of organ donors limit this option. In more advanced stages patients, chemotherapy is the only available treatment option; however, the traditional cytotoxic drugs affect both cancer and normal cells causing numerous side effects, including gastrointestinal tract ulcers, bone marrow depression, nausea, and hair loss [8]. As a result, neither current ablation therapies nor chemotherapy is appreciably effective in improving outcomes of this devastating disease. Considerable attention is needed for better therapy alternatives for hepatic cancer patients. A European study showed that prevention, development, progression, and treatment of cancers is associated with the diet of patients and a higher dietary intake of fruits and vegetables is associated with a lower risk of cancer development [7, 33]. There is always an increase in researchers' interest and efforts to identify new anticancer treatments with a scope of less toxic, more potent, more selective, and hence a more effective approach than other traditional ones [25].

Nature has been the best source of drugs since ancient ages; natural products are highly considered for their anticancer properties that do not only protect against cellular damage and disease but also have vital roles in the treatment of many diseases such as cancer [4, 17]. As a result of the aforementioned facts, it became necessary to

explore natural and plant-based products as a good substitute for chemotherapeutics to overwhelm the major adverse effects of chemotherapeutics, in addition to exhibiting improvements in therapeutic indices.

Punica granatum L. plant (Pomegranate) has been known for its nutritional values, medicinal properties and anticancer activities. Pomegranate parts such as peel, bark, root, and flower encompass various active phytochemicals. Among these parts, the pomegranate peel corresponds to around 50% of the fruit weight which is abundant in high molecular weight polyphenols including flavonoids, ellagic acids, gallotannins and ellagitannins (ETs) [50, 55]. ETs are the most bioactive components in pomegranates. Punicalagin (PU) is considered the most valuable and abundant ET in pomegranates [48]. The anticancer activity of pomegranate can be exerted in a chemo-preventive and/or chemotherapeutic approach. This activity is accountable for its anti-inflammatory, anti-invasive, anti-proliferative and pro-apoptotic activities in different types of cell lines such as skin, breast, prostate, colon and lung cancers [9]. Moreover, Bishayee et al. highlighted the remarkable chemo-preventive effect of PE against liver cancer using a chemical-induced and clinically relevant two-stage rat liver carcinogenesis model and ascribed this activity to the potent antioxidant and anticancer effects of PE extract [14].

Despite these promising characteristics and the intense therapeutic potency of PE extract, its poor solubility, instability and consequently its low bioavailability may hinder its applications. Nanotechnology has the unraveling ability to shield the PE extract inside the nanoparticles' (NPs) matrix and accordingly prevent additional degradation, and augment the bioavailability [13], in addition to its radical impact in achieving size-dependent passive targeting to specific organs relying on the enhanced permeability and retention (EPR) concept. To achieve this purpose, the particle size of NPs should be controlled to avoid the recognition by the reticuloendothelial system (RES) and to attain an efficient biodistribution pattern [34].

The employment of natural polymers for NPs preparation facilitates the accomplishment of safe dosage forms with low toxicity, high biocompatibility and biodegradability [26]. Many natural polymers such as collagen, zein and chitosan were extensively studied in previous research works [39]. Zein (ZN), a vegetable protein, is auspicious for the preparation of NPs due to its distinctive properties, including biocompatibility, biodegradability and reproducibility [45]. Its structure promotes the encapsulation of different bioactive compounds with hydrophobic, hydrophilic and amphiphilic characteristics, in addition to its desirable slow digestibility that imparts the formed NPs with long stability in the gastrointestinal tract before being degraded. In this regard, NPs that formed from zein have the suitability and ability to control the release of the encapsulated bioactive and to improve its bioavailability [5].

Hence, our aim is to incorporate PE peel extract into the natural biodegradable and biocompatible ZN NPs to provide an amenable green platform with a surfactant-free strategy able to deliver PE extract and ameliorate its activity against hepatic cancer. Utilization of such controlled system not only would result in augmenting the activity of PE but also would aid in the efficient liver targeting potential by the passive delivery in the future applications. The design and optimization were done using a $2^2 \times 3^1$ full factorial design to study the effect of the different variables on PE-ZN NPs characteristics. The physical properties of the developed NPs and the in vitro PE release from the NPs were investigated. Moreover, the ex vivo permeation of the PE-ZN NPs was examined as a prediction of the bioavailability of the investigated formula for the oral administration. Besides, the anti-proliferative activity (MTT assay) of the optimized formulation was studied in HepG-2 cancer cell lines in comparison with free PE.

Materials and methods

Materials

Pomegranate was purchased from Cairo Farms (Cairo, Egypt). Zein protein was generously obtained by FLO CHEMICAL CORPORATION (Wisconsin, USA) as a kind gift. Methanol was purchased from Sigma-Aldrich (St. Louis, USA). All other used chemicals were of analytical grade.

Cells

Human liver (HepG2) cells (VACSERA Company, Egypt) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) including 10% fetal bovine serum (Gibco, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 5% CO_2 atmosphere and temperature of 37 °C.

Preparation of pomegranate peel extract

Pomegranate fruits were cleaned up and washed with tap water; then dried and peeled. The peels were separated from the rind and cut into small pieces then dried at 50 °C in a drying oven (Heraeus, Germany) to dryness. The dried peels were ground to coarse powder of approximately 1 mm size. To prepare PE extract, 100 gm of ground pomegranate peel was soaked in 500 mL methanol and subjected to regular mixing for 6 h; followed by maceration for 48 h at 37 °C with intermittent shaking. The extraction procedure was repeated three times to extract the maximum components from the

pomegranate peel. Subsequently, the pooled extracts were filtered on Whatman No.1, UK filter paper. The filtrate was concentrated under vacuum using a rotary evaporator (Heidolph, Germany) at 50 °C to obtain 25 ml (mL). The concentrated extract was stored in a refrigerator at 4 °C [38, 40, 43].

Preliminary screening of surfactants

Surfactants of different hydrophilic-lipophilic balance (HLB), as shown in Fig. 1, were examined to investigate their effects on the PE encapsulation and the physical characteristics of the formed PE-ZN NPs. An amount of 50 mg of the surfactant was sonicated in 5 mL ethanolic dispersion of ZN in case of lipophilic surfactants (HLB < 7). For hydrophilic surfactants (HLB > 7), they were stirred in 10 mL preheated distilled water until complete dissolution with a magnetic stirrer (Stuart US150, USA). The PE-ZN NPs were prepared by the nanoprecipitation technique reported by [34]. Homogeneity of the formed dispersion and PE encapsulation efficiency were the two screening criteria for selecting the surfactant.

Quality target product profile (QTPP) and critical quality attributes (CQA)

As a prospective strategy to achieve the aim of the study effectively, a quality target product profile has been determined and is summarized in Fig. 2 with the desired quality attributes that will guarantee the achievement of the appropriate formula. The main target is to develop a green and safe dosage form against hepatic target. The employment of natural extract and natural polymer will be examined. The necessity of use of surfactant will be examined. The appropriate method of preparation that will yield the desired particle size for liver targeting and the adequate encapsulation efficiency with a controlled release, augmented ex vivo permeability and optimum activity will be studied. In addition the critical quality attributes that have a substantial role such as method of preparation, residual methanol, particle size, encapsulation efficiency, zeta potential, ex vivo permeability and in vitro cytotoxicity have been determined and justified as demonstrated in Fig. 2.

Factorial design

A $2^2 \times 3^1$ full factorial design was implemented to determine the effects of the selected variables using Minitab® 16.1.0 (Minitab Inc., USA). The three variables were: the dispersion pH (X1), PE: ZN ratio (w/w) (X2), and the surfactant concentration (mg/mL) (X3) using the selected surfactant from the preliminary study. The analysis of variance (ANOVA) was used to estimate the significant effects of the selected variables on the following

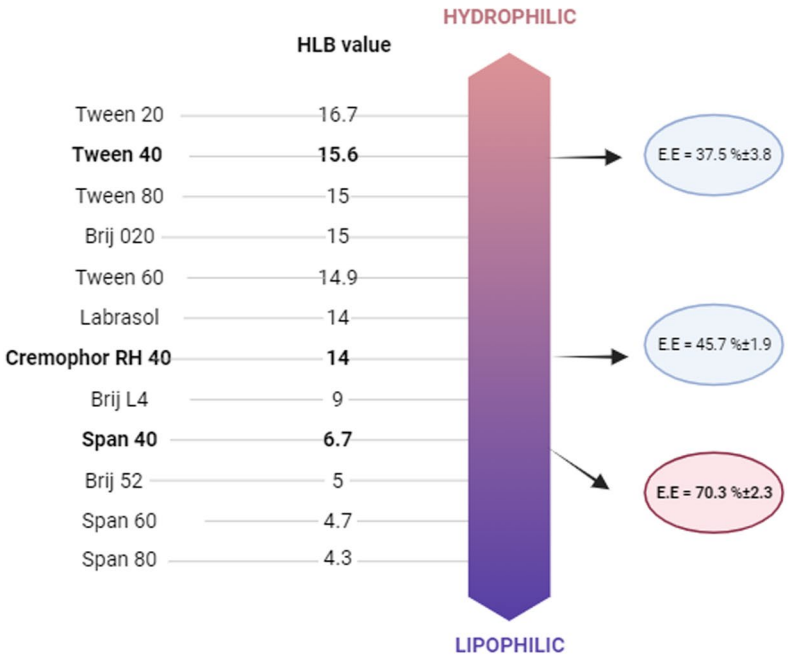


Fig. 1 Screened surfactants in the preliminary studies arranged according to their HLB

QTPP Element	Target	CQA	Justification
Dosage Form	Pomegranate peel extract based natural polymeric zein based nanoparticles		To afford a green and safe dosage form against hepatic cancer
Use of surfactant	Surfactant free		
Method of preparation	Nanoprecipitation	Yes	Affect the properties of the formed nanoparticles
Residual Methanol	To be below 3000ppm	Yes	For compliance with ICH Q3C
Particle size	Minimum Particle size <100 nm	Yes	To avoid the recognition by the reticuloendothelial system To achieve the size dependent liver targeting
Encapsulation Efficiency	Maximum encapsulation efficiency	Yes	To augment the extract activity and increase its stability
Zeta Potential	High zeta potential with a positive charge	Yes	To achieve stabilization and interaction with the negative cell membrane
In vitro release	Controlled release over 24 hours		
Ex vivo permeability	Higher permeability than the free PE	Yes	Prerequisite for oral administration
In vitro cytotoxicty	Considerable anti-prolerative activity against HepG2 liver cell line	Yes	Prerequisite for application in preclinical and clinical studies against hepatic cancer

Fig. 2 Quality target product profile (QTPP) and critical quality attributes (CQA)

responses; PE encapsulation efficiency (Y1), particle size (Y2), polydispersity index (PDI) (Y3) and zeta potential (Y4) of the PE-ZN NPs.

Preparation and characterization of PE-loaded ZN NPs

Preparation of PE-loaded ZN NPs

The nanoprecipitation procedure was utilized to prepare PE-ZN NPs; the steps are summarized in Fig. 3 with an asterisk on the critical process parameters (CPP). Separately, amounts of ZN and span 40 according to Table 1 were dissolved in 70% v/v ethanolic aqueous solution (5 mL) and PE extract was dissolved in methanol. After that, the two solutions were mixed and added drop wisely into a pH-adjusted aqueous phase (10 mL) (with either 0.1 N HCl or 0.1 N NaOH) under stirring with a magnetic stirrer (AccuPlate™ Analog Hot Plate Stirrer, UK) at room temperature and 2000 rpm for 1 h until the evaporation of alcoholic solvents.

Characterization of PE-loaded ZN NPs

Differential scanning calorimetry (DSC) A differential scanning calorimeter (Setline DSC+, Setaram, Switzerland) was utilized to scan the prospective physical incompatibilities between PE and the polymer or the surfactant in the dispersion. PE, physical mixtures of (PE and ZN) and (PE and the selected surfactant) were weighed in 30 µL Al-crucibles and measured in a range of 25–300 °C with heating rate of 10 °C/min. under nitrogen atmosphere [37].

Fourier transform infrared (FTIR) spectroscopy FTIR spectrophotometer (IR Affinity-1, Shimadzu, Japan) was used to evaluate the possible chemical interaction between PE and the polymer or the surfactant in the dispersion. Potassium bromide was physically mixed with the freeze-dried (Lyovapor L-200, Buchi, Switzerland) PE in addition to the physical mixture of ((PE and ZN) and (PE and the selected surfactant). Then a hydraulic pressing machine was used to compress these mixtures into thin discs. After that, the spectra were performed in the wavelength range from 400 to 4000 cm^{-1} [32].

Particle size (PS), polydispersity index (PDI) and Zeta potential (ZP) The average particle size accompanied by the polydispersity index (PDI) and zeta potential were evaluated by Zetasizer (Malvern Instruments Ltd, Malvern, UK). Samples were appropriately diluted with deionized water and all values were reported as the mean value ± standard deviation (SD) of three different measurements [1].

Encapsulation efficiency The centrifugation method was used to separate the free drug from PE-ZN NPs; the prepared dispersion of PE-ZN NPs was centrifuged at 10,000 rpm for 1 h at 4 °C using cooling microfuge

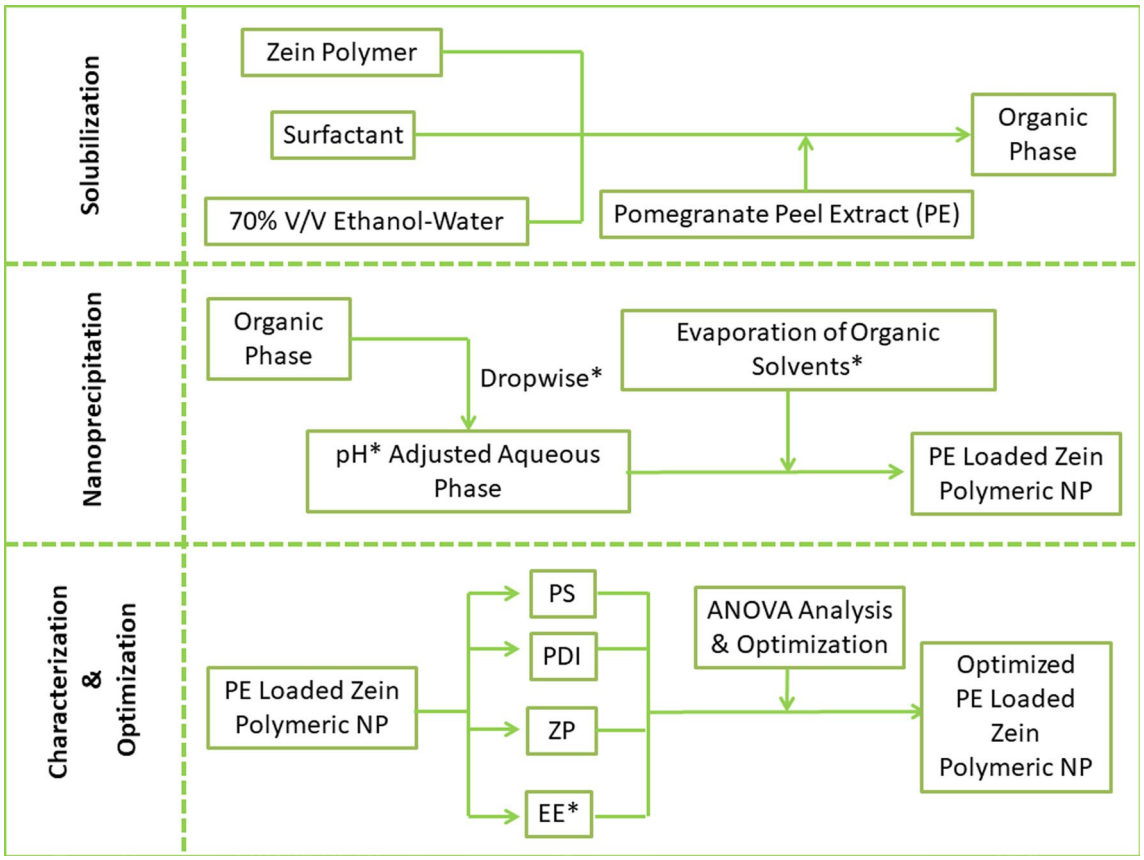


Fig. 3 Flowchart of preparation and characterization of PE ZN NPs

Table 1 Full factorial design independent and dependent variables and their levels

Independent variables	**Levels**		
	−1	**0**	**1**
X1: pH	2	7	9
X2: PE: ZN ratio (% W/W)	0.2		0.6
X3: Conc. of surfactant (mg/mL)	0		50
Dependent variable			**Constraints**
Y1: encapsulation efficiency			Maximize
Y2: particle size			Minimize
Y3: PDI			Minimize
Y4: zeta potential			Maximize

(Remi CM-12 Plus, Remi laboratory instruments, India). Then, the supernatant was diluted with methanol and measured spectrophotometrically at the wavelength of maximum absorbance (λmax) 265 nm using an ultraviolet–visible spectrophotometer (V-630, Jasco, Tokyo, Japan) in correspondence to a calibration curve of PE in methanol ($n=3$; $R^2=0.999$). PE encapsulation efficiency (EE%) was calculated by the following equation [9, 11]:

$$\text{PE encapsulation efficiency} = \frac{(\text{Initial amount of PE} - \text{amount of free PE}) \times 100}{\text{Initial amount of PE}} \quad (1)$$

Determination of residual solvents (methanol) Testing should be performed for residual solvents when production or purification processes are known to result in the presence of such solvents. As methanol (Class 2 Solvent) was used in the extraction of bioactive compounds from pomegranate peels, therefore the residual methanol was tested in the PE extract and the final optimum formula. The USP < 467 > protocol for determination of class 1 and class 2 residual solvents was utilized.

Morphological examination by transmission electron microscope (TEM) The morphology of the optimum NP formula was studied by TEM (JEM-2100, Japan). Each sample was negatively stained with phosphotungstic acid; the excess stain was removed by filter paper and then left to be air dried for 15 min. Consequently, a drop of the stained NPs was placed on a 200-mesh carbon-coated copper grid and the microscope was operated at an acceleration voltage of 200 kV [10].

In vitro drug release The dialysis membrane method was used to determine the PE release from the selected formulation and was compared to that of the free PE. Initially, the dialysis bags (MW cutoff of 12,000–14,000 Da) (Spectrum Medica, CA, USA) were soaked overnight in phosphate buffer solution (PBS) pH 7.4. The experiment was carried out using the USP dissolution (Hanson Research Corporation, CA, USA), apparatus II (paddle), at 37 °C, 100 rpm, and in 100 mL PBS pH 7.4 as a dissolution medium [9, 34]. A volume of 2 mL of the formula containing an amount of PE equivalent to 20 mg was placed in the dialysis bag and 2 mL aliquots were taken at predetermined time intervals and restored with fresh buffer to retain the sink condition. The same experiment was followed, but in this case 20 mg free PE/2 mL dissolution medium was placed in the dialysis membrane. A preliminary study was done to ensure that the above conditions provide a sink medium for PE at this dose level. PE concentration in the samples was assessed spectrophotometrically at 265 nm in correspondence to a calibration curve of PE in PBS pH 7.4 ($n = 3$; $R^2 = 0.999$).

Determination of release kinetics To determine the release of PE extract from the optimized NP preparation, (zero order, first order and Higuchi) mathematical kinetic models have been employed for the in vitro drug release results. The coefficients of determination (R^2) results of the three models for the optimum preparation were calculated [28].

Ex vivo permeability study Ex vivo permeability of the investigated formula was done to investigate the permeability parameters of pure free PE extract and optimized PE-ZN NPs (Z12) formulation. Small intestines of rabbits that were allowed to fast overnight were used. The duodenal parts were isolated and their contents were removed by flushing with normal saline. Subsequently, they were divided into segment sacs of 5 cm and a volume of 2 mL of the optimum formula containing an amount of PE equivalent to 20 mg was placed in each sac which their ends were tied carefully with sutures [36]. The same was done for free PE and blank ZN NPs (free from PE) to act as a blank. A USP dissolution with amber mini-vessels (Hanson Research Corporation, CA, USA), was used and each sac was tied onto apparatus II (paddle), at 37 °C, 75 rpm, and in 100 mL PBS pH 7.4 as a dissolution medium. Samples of 1 mL were withdrawn at time intervals of (0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h) and replaced with an equivalent amount of the fresh buffer solution. The samples were assayed spectrophotometrically at 265 nm [52] with a calibration curve of PE in PBS pH 7.4 ($n = 3$; $R^2 = 0.998$), and the experiment was repeated in triplicate.

In vitro cytotoxicity study

The cytotoxic effect of both the optimized PE-ZN NPs formula and the free PE on HepG2 cancer cell lines (ATCC® HB-8065™) was studied. Samples of free PE and optimized formulae were diluted on pre-cultured cell lines for 48 h treatment at 37 °C post-decanting growth medium. Treated cell lines were microscopically examined for detection of morphological changes and detached cells. Dead cells were washed-out using phosphate buffer saline (PBS), pH 7.2 ± 0.2 (with 0.05% Tween 20). Residual live cells were treated with 0.5% MTT stain as 25 µL/well. Plates were incubated for 3–4 h at 37 °C. Developed intra-cytoplasmic MTT formazan crystals were dissolved using 0.05 mL DMSO for 30 min on a plate shaker. Optical densities (OD) were read using an ELISA plate reader (Anthos-Elisa-reader 2001, Labtec, Heerhugowaard, Netherlands). The 50% inhibitory concentration (IC_{50}) of both the PE extract and the optimized PE-ZN NPs formula were determined using the Master–plex-2010 program (Hitachi Software Engineering America, Ltd). Data were recorded for three independent experiments. The viability percentage was calculated as follows [6, 54]:

$$\text{Cell viability percentage} = (\text{OD of treated cells}/\text{OD of untreated cells}) \times 100. \quad (2)$$

Results

Preliminary screening of surfactants

The screened surfactants were subjected initially to physical inspection. Only formulations prepared with Tween® 40, Cremophor® RH 40, and Span® 40 displayed homogeneous dispersions, while those formulated with other surfactants showed precipitates. Afterward, the homogenous formulations were exposed to another screening step of estimation of the PE encapsulation efficiency. As shown in Fig. 1 Span® 40 formula revealed a remarkably higher PE encapsulation efficiency of 70.3% ± 2.3 in comparison with 45.7% ± 1.9 for Cremophor® RH 40 formula and 37.5% ± 3.8 in case of Tween® 40. This may be attributed to that zein is a polymer having three-fourths lipophilic and one-fourth hydrophilic amino acids residues, thus acting as a water barrier, and helping in attaining higher encapsulation efficiency values of PE in NPs, especially those including Span® 40. That verifies that there is a reversible relationship between the PE encapsulation and the HLB values of the used surfactant during ZN NPs formation Therefore, Span 40 was selected to be utilized in further studies [12, 23].

Characterization of PE-loaded ZN NPs

Differential scanning calorimetry (DSC)

As shown in Fig. 4 and supplemental Fig. 1A, B and C, the DSC thermogram of the PE showed a sharp endothermic peak at a temperature of 142.791 °C that can be related to the melting point of polyphenols present in PE [56]. The physical mixture of PE: ZN thermogram had the same peak with a slight shift to 146.047 °C with no other peaks for ZN similar to that reported in previous study [45]. It is worth noting that the mixture with polymers could enhance the thermal stability of PE resulting in this slight higher melting temperature [30]. On the other hand, it is depicted from the thermogram of the physical mixture of PE: Span® 40 the appearance of two endothermic peaks at 56.394 °C and 115.666 °C. The first endothermic peak could be attributed to the melting point of Span® 40 as reported in literature [47]. The another peak could be assigned to the melting of PE and this shift to lower temperature can be justified to the result of the partial dissolution of PE in the physical mixture of PE and Span® 40 when the latter is heated [42].

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the freeze-dried PE and the two physical mixtures of PE with ZN and Span 40 as shown in Fig. 5 revealed the wide band recorded at 3346 cm^{-1} corresponding to the stretching vibration of N–H and O–H groups and the peaks between 1046 to 1800 cm^{-1} belonged to the aromatic and aliphatic functional groups of the polyphenolic content of PE including CH, C=O, –C=C–C=O, –C=C– and –C–C–. The investigated spectra showed an insignificant shift in the peaks of PE in the physical mixtures relative to the peaks in the spectrum of the pure extract, indicating the absence of any interactions between PE and the other used excipients. The reduction of the peak intensity of PE in the physical mixture may be due to the dilution effect of the mixing process [51]. Therefore, PE and all the used excipients are compatible to be used collectively.

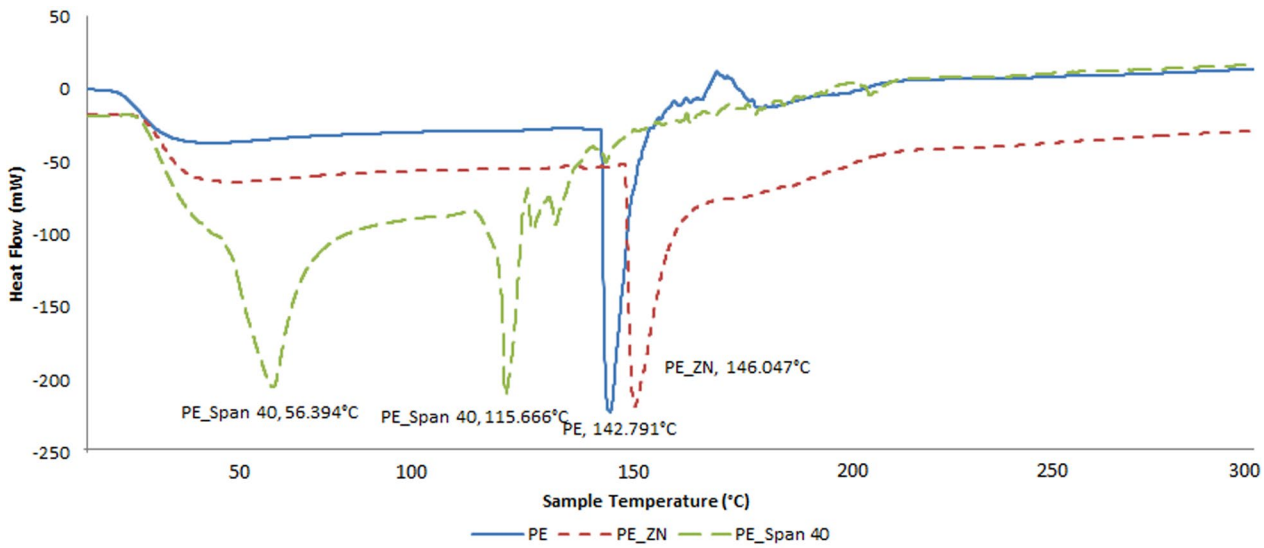


Fig. 4 DSC thermograms of PE and the physical mixtures of PE with ZN and span 40

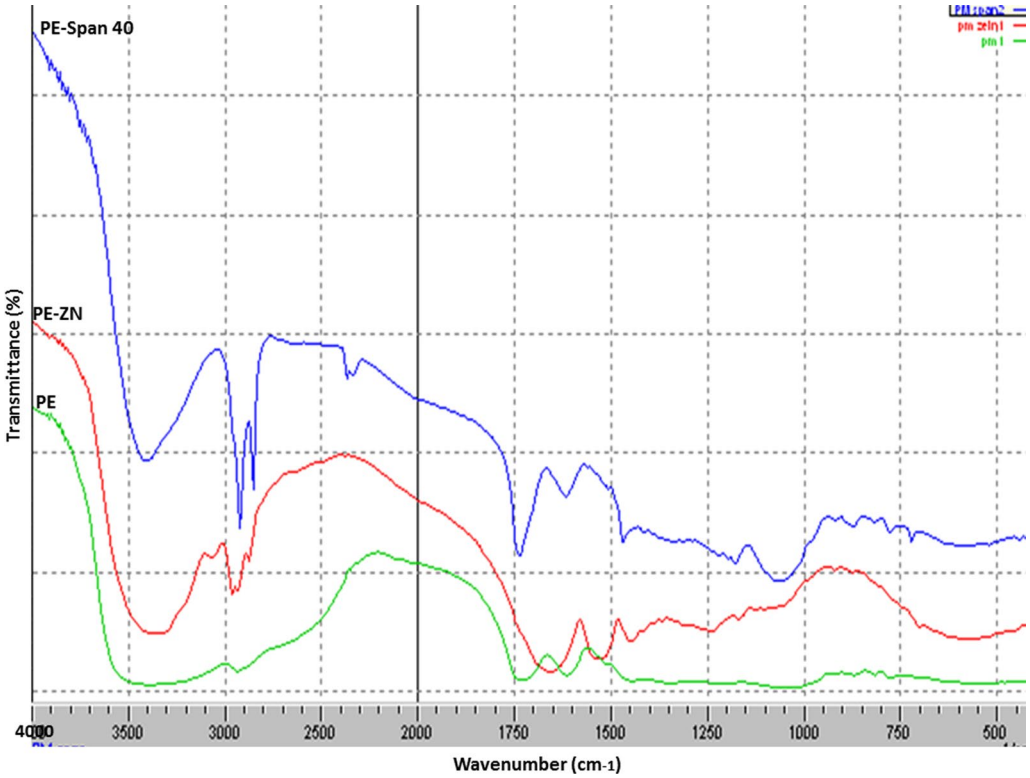


Fig. 5 Fourier transform infrared spectra of freeze-dried PE and the physical mixtures of PE with ZN and span 40, respectively

Table 2 Preparations and characterization of PE-ZN NPs

#	pH	PM: ZN (% W/W)	Surfactant (mg/mL)	EE (%)	PS (nm)	PDI	ZP (mV)
Z1	7	0.6	50	64.46 ± 1.23	973.1 ± 9.61	0.869 ± 0.001	−8.8 ± 0.7
Z2	7	0.6	0	80.84 ± 3.56	206.7 ± 7.80	0.129 ± 0.003	32.5 ± 1.2
Z3	7	0.2	0	73.4 ± 0.89	185.0 ± 8.90	0.314 ± 0.002	43.3 ± 2.3
Z4	7	0.2	50	68.36 ± 5.62	1335.0 ± 1.31	0.593 ± 0.002	−22.4 ± 0.6
Z5	9	0.2	0	99.12 ± 2.89	175.0 ± 6.77	0.103 ± 0.001	−37.9 ± 1.5
Z6	9	0.6	50	99.57 ± 1.78	116.2 ± 14.60	0.411 ± 0.001	17.2 ± 2.4
Z7	9	0.2	50	99.05 ± 6.59	152.9 ± 10.31	0.497 ± 0.002	17.6 ± 0.8
Z8	9	0.6	0	99.14 ± 0.23	173.8 ± 9.55	0.096 ± 0.001	−41.4 ± 0.2
Z9	2	0.6	50	99.3 ± 4.23	177.2 ± 5.67	0.101 ± 0.003	46.5 ± 1.7
Z10	2	0.2	50	99.32 ± 1.56	278.1 ± 7.37	0.408 ± 0.001	44.9 ± 2.6
Z11	2	0.6	0	99.29 ± 0.56	131.0 ± 2.54	0.149 ± 0.002	24.8 ± 0.1
Z12	2	0.2	0	99.31 ± 3.64	99.5 ± 6.34	0.237 ± 0.001	37.8 ± 1.3

Dependent variables are reported as average values ($n = 3$) ± SD

Particle size (PS), polydispersity index (PDI) and Zeta potential (ZP)

The average particle size (PS) values of the obtained PE-ZN NPs ranged from 99.5 ± 6.43 nm (Z12) to 1335.0 ± 1.31 nm (Z4) as shown in Table 2 and supplemental Fig. 5. The ANOVA analysis showed a significant effect of the 2 variables (X1; pH and X2; surfactant concentration) (p-values of 0.015 and 0.023, respectively) on the PS, while PE: ZN ratio showed a non-significant effect (p-value > 0.05). PS was noticed to be high at the pH of the dispersion near the isoelectric point (PI) of ZN which might be attributed to the aggregation of the colloidal dispersion when the pH gets close to the PI of ZN polymer. Accordingly, the PS was shown to be reduced

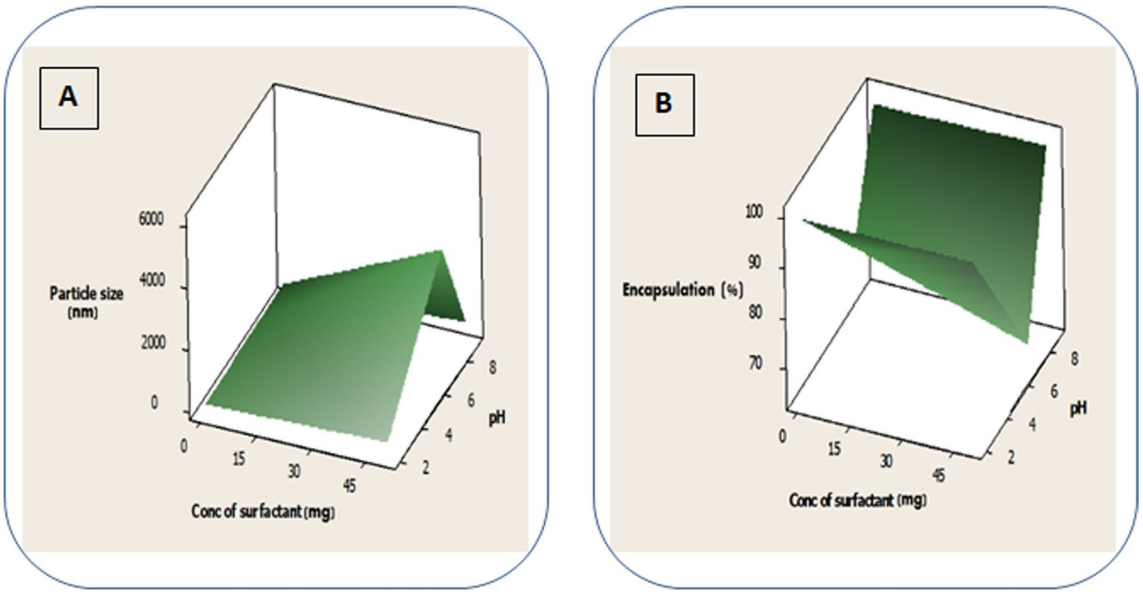


Fig. 6 Response surface plot of effect of **A** dispersion pH and **B** conc. of surfactant on particle size

apart from PI at both pH 2 and pH 9 as shown in Fig. 6A. The incorporation of a surfactant led to an increase in PS. This was ascribed to the fact that surfactants with high and low HLB values are not able to be adsorbed on hydrophobic surfaces of drug particles which led to the aggregation of these particles to agglomerate rapidly increasing the particle size [46]. The PDI values of the obtained PE-ZN NPs alternated from 0.096 to 0.869, indicating a narrow size distribution, since all values were less than 1. The concentration of the surfactant (X3) showed a significant effect on PDI (p-value < 0.05). Formulations free of surfactants showed more homogenous distribution than those containing a surfactant. On the contrary, the other two factors (PE: ZN ratio and pH) showed a non-significant effect on PDI. In the present study, the zeta potential of PE-ZN NPs showed a relatively high zeta potential except Z1 which showed the lowest zeta potential (-8.8 ± 0.7) as shown in Table 2. This may be attributed to the PS observed of Z1 (973.1 ± 89.2 nm) where the attraction between particles may exceed the electrostatic repulsive forces, so the zeta potential decreased and the PS increased [31]. Zeta potential at pH lower than ZN PI (pH 6.8) exhibited a positive charge. However, at PI, the dispersion showed a zeta potential of negative value in the presence of nonionic surfactant (Span® 40). It was stated that at a neutral pH, many nonionic surfactants granted a negative charge on the NPs as a result of the differential adsorption of the produced ions (H_3O^+ and OH^-) on their surfaces [2, 31]. On the other side, Podaralla and Peruma indicated that at high alkaline pH, a very high variation in the zeta potential of the ZN NPs is expected. This variation was observed in formulations prepared at pH 9 [44].

Encapsulation efficiency

EE% was determined by measuring the amount of unencapsulated extract in the aqueous phase after centrifugation of the developed dispersion systems as displayed in Table 2. It was observed that ZN-NPs entrapped a significant amount of PE (64.46–99.57%). pH significantly affected the PE encapsulation efficiency (p value < 0.05). pH apart from the isoelectric point (PI) of ZN protein polymer, such as pH 2 and pH 9 showed a high amount of encapsulated extract in contrast to pH 7 (isoelectric point of ZN) which showed a lower amount of PE entrapped. McTigue and Perry observed an increase in the encapsulation efficiency of the investigated protein (Hen Egg White Lysozyme) while using a pH away from its PI [15]. On the other side, the other two factors (PE-ZN ratio and surfactant concentration) showed a non-significant effect on the EE as displayed in Fig. 6B.

Selection of the optimum ZN NP preparation

Selection of the optimum preparation was based on studying the effects of the different preparation variables on the four essential investigated responses (EE%, PS, PDI and ZP). According to the ANOVA analysis, pH and surfactant concentration have a principal significant effect on EE% and PS, respectively. It is inferred that pH apart from the PI of ZN was favorable to obtain high EE% and small PS. In addition, increasing the surfactant concentration from zero to 50 mg/mL had a negative effect on

PS. Therefore, preparations with a surfactant concentration level of 50 mg/mL were excluded. On the other hand, the priority was attained to NP preparations of pH 2. Basically for two reasons: first, to avoid the obtained variations in ZP at pH 9 and second, to obtain NP with a positively charged surface for the favorable interaction with the negatively charged cell membrane. By the examination of the NP preparations of pH 2 level, the preparation of the maximum EE%, minimum PS and highest positive ZP was obtained by Z12. Therefore, Z12 was selected as the optimized NP preparation and subjected to further characterization.

Determination of residual solvents (methanol)

The determination of methanol as a residual solvent in pharmaceuticals is an important quality control measure. Methanol is classified as a Class 2 solvent which should be limited due to potential toxicity. The compendial method for determination of residual solvents USP < 467 > was capable to determine the residual methanol in both pure PE extract and the optimized formulation of PE-ZN NPs (Z12). The results were 812 ppm and 237 ppm for the pure PE extract and the optimized formulation (Z12), respectively. According to ICH Q3C (R8) option 1, the acceptable amount of methanol as a residual solvent in the final product should be not more than 3000 ppm based on 30 mg/day permitted daily exposure (PDE) and daily dose does not exceed 10 g. The two results were below the limit indicating the safety of the formula and the compliance with global regulatory requirements.

Morphological examination by transmission electron microscope (TEM)

The examination of the morphology of the optimized formulation of PE-ZN NPs (Z12) under transmission electron microscope showed that the ZN-NPs had a spherical uniform shape and a smooth surface. There was no aggregation, and the size of the particles was in the nanosize, matching the results obtained by the Zetasizer as shown in Fig. 7.

In vitro drug release

The optimized ZN NP preparation (Z12) was selected to study its release profile in comparison with free PE extract. The free extract showed a rapid release pattern of more than 75% of PE within 2 h as inferred from Fig. 8, while the release pattern of PE from the optimum preparation was relatively slow. This slow release pattern was confirmed by the significant difference (p value < 0.0001)

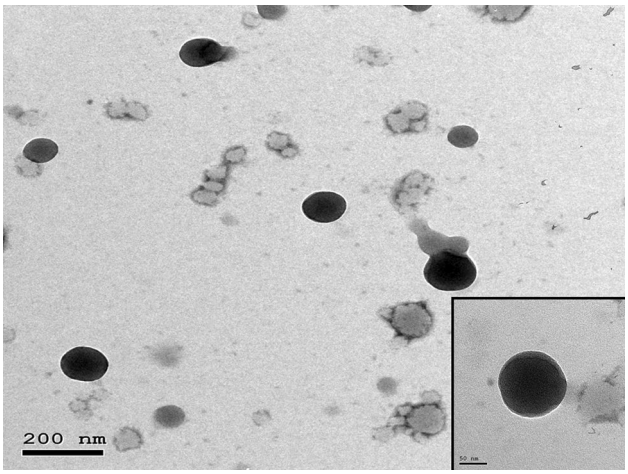


Fig. 7 TEM image of the optimized PE ZN NP

in release between free PE extract and Z12 at 2 h. Moreover, it achieves a controlled release over 24 h with higher stability of the encapsulated PE in Z12 comparable to the obvious decline in the release of PE (p value < 0.0011) in the period between 10 and 24 h which most probably might be justified by the degradation of the released free PE in the release medium [9].

Determination of release kinetics

The results of the estimation of (R^2) of the utilized three mathematical models revealed that the highest (R^2) was for the Higuchi model pointing to a diffusion-controlled release of the encapsulated PE extract from the hydrophobic matrix of the formed ZN NPs to the external aqueous phase.

Ex vivo permeability study

As illustrated in Fig. 9, the ex vivo cumulative permeation percentages of PE through the rabbit intestine from both optimized PE-ZN NPs (Z12) and free PE extract over 24 h were plotted. It was noticeable that the permeation percentages from both optimized PE-ZN NPs (Z12) and free PE were comparable till the first hour. Thereafter, the permeation rate of free PE was relatively slower than the optimized PE-ZN NPs (Z12) and reached the maximum at 6 h with cumulative percentage of (56.96% ± 1.72) and significant difference (p value < 0.0133) from the optimized Z12 formula that had permeation percentage of (63.62 ± 3.54). Conversely, the permeation of PE from the optimized PE-ZN NPs (Z12) was continuous after 6 h and reached the maximum at 24 h with cumulative permeation percentage of (89.02% ± 4.58). This relative increment in PE permeation in case of PE-ZN NPs could be attributed to several factors. For instance, the

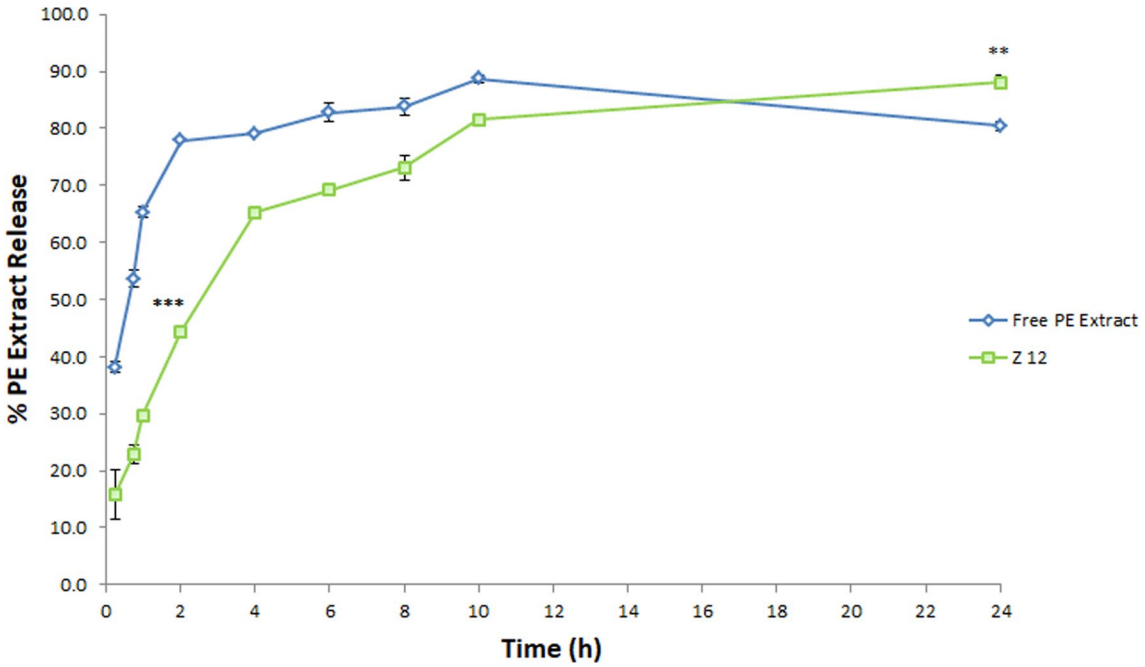


Fig. 8 In vitro release of pomegranate from PE ZN NPs compared to PE

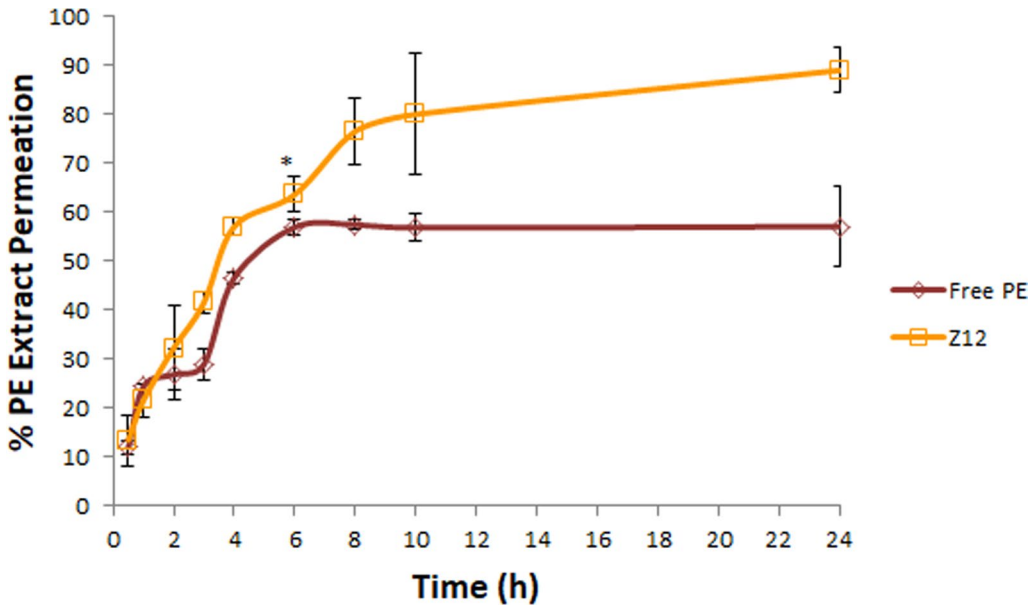


Fig. 9 Ex vivo permeation of pomegranate from PE ZN NPs compared to PE

small nanosized of the particles and the high positive charge of the NPs surface that aid to increase the interaction with the cell membrane and consequently augment the flux and permeation of the extract. In addition, the hydrophobic properties of the zein molecules due to the γ-zein N-terminal repetitive part promote the extract permeation through interaction with the cell membranes components. Similar finding were reported by [21, 24].

In vitro cytotoxicity study

To examine the effect of nanoencapsulation on the biological activity of PE, the cytotoxic effect of the optimized

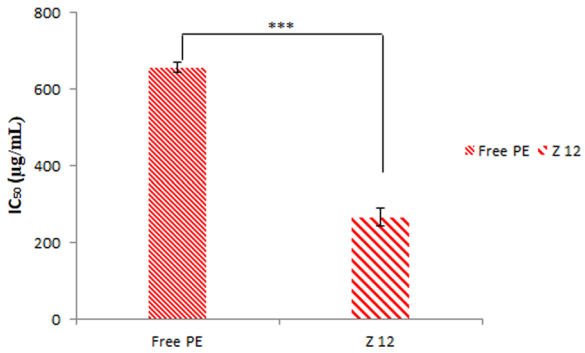


Fig. 10 IC50 values of the PE extract and PE ZN NPs (Z12)

preparation of PE-ZN NPs (Z12) and the free PE on HepG2 cancer cells were compared. It was found that the optimized formula (Z12) had a superior anti-proliferative effect on the HepG2 liver cell line with a 50% inhibitory concentration (IC50) of 266.68 µg/mL ± 23.11 relative to 657.47 µg/mL ± 13.84 in case of free PE extract. As demonstrated in Fig. 10, the optimized PE-ZN NP preparation (Z12) increased the potency of the cytotoxic activity on HepG2 hepatic cancer cells by reducing the IC50 values by about 2.5-fold than the free PE (p value < 0.0001). This finding may be attributed to the more interaction between the negatively charged cell membranes and the positive charge of the optimized preparation [3]. Furthermore, the augmented cytotoxic activity might be due to the high ability of PE NP in internalization of PE into the cells relative to the free extract [36].

Discussion

To sum up, hepatic cancer with its high morbidity and lethality increases the emergence of developing promising platforms to combat it. Nature is a precious source of natural products that have proven their medicinal properties. Considerable anticancer therapeutics have originated from natural sources. Phytochemicals and their valuable constituents always pave the way to handle chronic health threats. Pomegranate is an ancient fruit with auspicious antioxidant and anticancer properties and is considered an effective remedy for different cancer diseases. Punicalagin is one of the most valuable polyphenols in pomegranates which is not absorbed in its intact form but hydrolyzed into moieties of ellagic acid and rapidly metabolized into short-lived metabolites of ellagic acid [48].These compounds possess many biological activities in the prevention and treatment of cancer [29, 53] because of their free radical scavenging activities [22] as well as their anticancer effects [48].

Accordingly, our goal in this study is to make a formula that stands out for the therapeutic significance of pomegranate and overcome any challenges that may affect the physicochemical properties, bioavailability and stability of pomegranate. Many attempts have been made to augment its biological activities and studies have been performed to assess the safety and efficacy. For example, Shirode et al. [48] have developed poly (d,l-lactic-*co*-glycolic acid)–poly(ethylene glycol) NPs encapsulated with PE extract with efficient cellular uptake and anti-proliferative activity against different breast cancer cell lines. Another study obtained by Badawi et al. [9] who optimized solid lipid nanoparticles loaded with PE extract and improved its cytotoxic activity against different cancer cell lines relative to the free PE extract. Finally, Soltanzadeh et al. [49] encapsulated the PE extract in chitosan NPs aiming to protect its sensitive constituents but a limitation to encapsulation has been observed when increasing the PE concentration.

In our study, we focused on increasing the encapsulation efficiency, controlling the release of PE extract and achieving a qualified platform suitable for targeted delivery to hepatic cancers. Zein natural polymer was utilized to load PE extract by carrying on a $2^2 \times 3^1$ full factorial design to study the effect of different variables on the properties of the formed NPs.

The screening of the potential physical or chemical incompatibilities as a prerequisite for selection of the most suitable excipients in the dosage form was done by implementing two concomitantly and complementary analytical techniques, namely DSC and FTIR [18]. The FTIR analysis showed the chemical compatibility between the PE extract and the selected polymer and surfactant and the DSC analysis indicated the presence of the sharp melting peak of PE in the DSC of the physical mixtures of both zein polymer and span® 40 with a forward shift in case of zein due to the thermal stability and a backward shift in case of span® 40 on account of the partial dissolution of PE in the melted surfactant.

The pH of dispersion at three levels, namely 2, 7 and 9, parallel with two levels of Span 40 as surfactant concentrations, namely zero and 50 mg/mL were selected to demonstrate the feasibility to rely on tuning the pH of the dispersion and passing up the use of surfactants. The ANOVA analysis revealed the significant effect of pH on PS (being high at pH 7 near the isoelectric point (PI) of ZN and low at pH apart from PI at both pH 2&9), EE% (pH 2&9 showed a high amount of encapsulated extract in contrast to pH 7) and ZP. While the surfactant concentration had a significant effect on PS and PDI, the change of PE: ZN ratio had no significance on any of the investigated responses. The observed effects on each response were in harmony with literature. In detail, the observed effect of pH on PS has been reported by many authors such as [44]

that reported that the decrease in PS of ZN NPs is due to the existence of zein polymer in in the monomeric form at pH > PI. On the other hand, [19] attributed the low PS and high porosity of ZN NPs at low pH to the more solubility of zein in acidic pH. For the encapsulation efficiency, the attained EE% at pH apart from the PI was remarkably high around 99%, on the other hand, a noticeable decrease in the EE% at pH 7 (near the PI) from 64.46 ± 1.23 to 80.84 ± 3.56%. This could be appropriately attributed to the refolding behavior of proteins at the PI which could result in the escape of the encapsulated extract leading to less encapsulation efficiency than that obtained at pH far from the PI as reported by [35]. In case of the ZP, the effect of pH on the ZP of the prepared ZN NPs was in agreement with [27] who attributed the obtained change in the ZP of the uncoated ZN NPs from highly positive at low pH to highly negative at high pH to the electrical characteristics dominated by the zein molecules at particles' surfaces, which are cationic at low pH due to protonation of amino and carboxyl groups ($-NH_3^+$ and $-COOH$) and anionic at high pH due to de-protonation of these groups ($-NH_2$ and $-COO^-$). Finally, the surfactant concentration effect was negative on both PS and PDI with observable increase in PS and less homogeneity with relatively higher PDI values than the surfactant-free formulations. Based on these findings, it is inferable that the integral positive effect of changing the pH of the dispersion medium in comparison of the incorporation of surfactant and the adequacy of the hypothesis of the reliance on tuning the dispersion pH is deduced.

As per the predetermined QTPP and the above ANOVA analysis, the optimization resulted in an optimized ZN NP preparation having a small PS of 99.5 ± 6.43 nm (< 100 nm) that a prerequisite for size-dependent passive targeting to liver as reported by [34] who studied the distribution of 5-fluorouracil zein nanoparticles and discussed the optimum average size required for accumulation in liver after IV administration taking into consideration the fenestrae size and the necessity of the unrecognition by RES and high EE% of 99.31 ± 3.64. In addition, a PDI value of 0.237 ± 0.001 indicates narrow size distribution and a high positive ZP charge of 37.8 ± 1.3 that imports good physical stability and ability to interact efficiently with negatively charged cell membranes. Then the performed in-vitro study on the optimized ZN NP preparation showed a distinguishable and stable in vitro release pattern comparable to the free PE extract in a controlled manner for 24 h achieving sustained release profile. Additionally, as the methanol which is a class 2 solvent was used in the PE extract preparation and it is known that residual solvents have no therapeutic value and may affect physicochemical properties of the active constituents and have potential toxicity [20]. Taking into consideration exceeded quantities of such solvents can consequently result in risks for patients. Methanol (Permissible Daily Exposure 30 mg/day) can be used in the pharmaceutical products with permissible limit of 3000 ppm as a residual solvent in the final products according to ICH guideline for Residual Solvents Q3C (R9). GC analysis was performed to detect the quantity of the residual solvent in both the PE extract and the optimized NPs. The obtained results showed that both the extract and the formula were below the permissible limit indicating the safety of the formula and the compliance with global regulatory requirements.

Nanosized formulations exhibit a large loading capacity together with a larger surface on which the loaded drug can conjugate with targeting organs with a controlled permeation pattern [41].This was exhibited through the ex vivo permeability study that has revealed an enhancement of 1.5 folds of the cumulative PE permeation from the optimized ZN NP over 24 h in comparison with PE extract that delivered its content through only 6 h.

Oxidative stress is one of the main causes of the development of human hepatic cancer. Bishayee et al. [14] demonstrated the potential effect of using pomegranate products in the chemoprevention and treatment of hepatic cancer through overcoming the oxidative damage trough modulation of Nrf2 signaling. In our study, the anti-proliferative activity (MTT assay) of the optimized formulation was studied in HepG-2 cancer cell lines in comparison with free PE. The potential of the optimized preparation to enhance the cytotoxic activity of PE extract was evidenced by an in vitro cytotoxicity study that revealed the superiority of PE-loaded ZN NP over Free PE extract on HepG2 liver cell line by significantly decreasing the IC50 by 2.5 fold than the free PE which in turn proves our endeavor and presents a promising platform for future utilization in preclinical and clinical studies against hepatic cancer.

Conclusion

Successful treatment for hepatic cancer was aimed in this study by loading pomegranate peel extract in nanosized particles with the aid of zein polymer which achieves high drug encapsulation efficiency with small particle size. This developed system attained more effective and more retardation of pomegranate extract release than the free extract. The in vitro drug release study demonstrated that the free extract showed a high percentage of release of about 80% within 2 h, while the optimized preparation exhibited more retardation till 24 h. The in vitro cytotoxicity study showed that PE-ZN NPs attained reduced IC_{50} values by 2.5-fold than the free PE; thus, formulated pomegranate extract in zein nanoparticles has a more

effective and cytotoxic effect than free extract. The study demonstrated that the pomegranate zein nanoparticles could be a promising green drug delivery system, where both the active ingredient and the carrier polymer are derived from plants with a surfactant-free strategy, thus protecting the liver against toxins and can be safely and effectively used to treat hepatic cancer. Finally, future stability studies, more in vitro tests to understand the underlying mechanisms of the hepatic anticancer properties of prepared NPs of the optimum formula and in vivo experiments are required to validate PE-ZN NPs efficacy and bioavailability. Consequently, the approach may be worth considering for scale-up to expand its applications in clinical studies against hepatic cancer.

Abbreviations

ANOVA	Analysis of variance
CPP	Critical process parameters
CQA	Critical quality attributes
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
EE	Encapsulation efficiency
EPR	Enhanced permeability and retention
ETs	Ellagitannins
FTIR	Fourier transform infrared (FTIR) spectroscopy
HLB	Hydrophilic lipophilic balance
ICH	International Council on Harmonization
IC50	The 50% inhibitory concentration
NPs	Nanoparticles
Nrf2	Nuclear factor erythroid 2-related factor 2
OD	Optical density
PBS	Phosphate buffer solution
PDI	Polydispersity index
PE	Pomegranate extract
PI	The isoelectric point
PS	Particle size
PU	Punicalagin
QTPP	Quality target product profile
RES	Reticuloendothelial system
RPM	Rotate per minute
SD	Standard deviation
TEM	Transmission electron microscope
ZP	Zeta potential
ZN	Zein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43094-024-00647-9.

Supplementary Material 1.

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Author contributions
S.M. contributed to the conceptualization. D.M.N.A. and M.M.B. was involved in the methodology and experimental design; S.M. assisted in screening, formulation, and characterization. M.A.E. was involved in the plant extraction. S.M. and M.M.B. prepared the original draft literature. D.M.N.A. contributed to the discussion. A.N.E. and D.M.N.A. contributed to the review and editing. A.N.E., D.M.N.A. and A.R.F. were involved in the supervision.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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References

1. Abdel-All SR, Shakour ZTA, Abouhussein DMN, Reda E, Sallam TF, El-Hefnawy HM, Abdel-Monem AR (2021) Phytochemical and biological evaluation of a newly designed nutraceutical self-nanoemulsifying self-nanosuspension for protection and treatment of cisplatin induced testicular toxicity in male rats. Molecules 26(2):408. https://doi.org/10.3390/molecules26020408
2. Abouhussein DMN, Bahaa El Din Mahmoud D, Mohammad FE (2019) Design of a liquid nano-sized drug delivery system with enhanced solubility of rivaroxaban for venous thromboembolism management in paediatric patients and emergency cases. J Liposome Res 29(4):399–412. https://doi.org/10.1080/08982104.2019.1576732
3. Ali MS, Metwally AA, Fahmy RH, Osman R (2020) Chitosan-coated nanodiamonds: mucoadhesive platform for intravesical delivery of doxorubicin. Carbohyd Polym 245:116528. https://doi.org/10.1016/j.carbpol.2020.116528
4. Alok S, Jain SK, Verma A, Kumar M, Mahor A, Sabharwal M (2014) Herbal antioxidant in clinical practice: a review. Asian Pac J Trop Biomed 4(1):78–84. https://doi.org/10.1016/S2221-1691(14)60213-6
5. André de Almeida Campos L, Francisco Silva Neto A, Cecília Souza Noronha M, Ferreira de Lima M, Macário Ferro Cavalcanti I, Stela Santos-Magalhães N (2023) Zein nanoparticles for drug delivery: preparation methods and biological applications. Int J Pharm 635:122754. https://doi.org/10.1016/j.ijpharm.2023.122754
6. Anitha A, Deepagan VG, Divya Rani VV, Menon D, Nair SV, Jayakumar R (2011) Preparation, characterization, in vitro drug release and biological studies of curcumin loaded dextran sulphate–chitosan nanoparticles. Carbohyd Polym 84(3):1158–1164. https://doi.org/10.1016/j.carbpol.2011.01.005
7. Anwanwan D, Singh SK, Singh S, Saikam V, Singh R (2020) Challenges in liver cancer and possible treatment approaches. Biochim Biophys Acta Rev Cancer 1873(1):188314. https://doi.org/10.1016/j.bbcan.2019.188314
8. Aswad M, Rayan M, Abu-Lafi S, Falah M, Raiyn J, Abdallah Z, Rayan A (2018) Nature is the best source of anti-inflammatory drugs: indexing natural products for their anti-inflammatory bioactivity. Inflamm Res 67(1):67–75. https://doi.org/10.1007/s00011-017-1096-5
9. Badawi N, Teaima M, El-Say K, Attia D, El Nabarawi M, Elmazar M (2018) Pomegranate extract-loaded solid lipid nanoparticles: design, optimization, and in vitro cytotoxicity study. Int J Nanomed 13:1313–1326. https://doi.org/10.2147/IJN.S154033

10. Bakr MM, Shukr MH, ElMeshad AN (2020) In situ hexosomal gel as a promising tool to ameliorate the transnasal brain delivery of vinpocetine: central composite optimization and in vivo biodistribution. J Pharm Sci 109(7):2213–2223. https://doi.org/10.1016/j.xphs.2020.03.030
11. Baspinar Y, Üstündas M, Bayraktar O, Sezgin C (2018) Curcumin and piperine loaded zein-chitosan nanoparticles: development and in-vitro characterisation. Saudi Pharm J 26(3):323–334. https://doi.org/10.1016/j.jsps.2018.01.010
12. Bayindir ZS, Yuksel N (2010) Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery. J Pharm Sci 99(4):2049–2060. https://doi.org/10.1002/jps.21944
13. Bharali DJ, Siddiqui IA, Adhami VM, Chamcheu JC, Aldahmash AM, Mukhtar H, Mousa SA (2011) Nanoparticle delivery of natural products in the prevention and treatment of cancers: current status and future prospects. Cancers 3(4):4024–4045. https://doi.org/10.3390/cancers3044024
14. Bishayee A, Bhatia D, Thoppil RJ, Darvesh AS, Nevo E, Lansky EP (2011) Pomegranate-mediated chemoprevention of experimental hepatocarcinogenesis involves Nrf2-regulated antioxidant mechanisms. Carcinogenesis 32(6):888–896. https://doi.org/10.1093/carcin/bgr045
15. Blocher McTigue WC, Perry SL (2019) Design rules for encapsulating proteins into complex coacervates. Soft Matter 15(15):3089–3103. https://doi.org/10.1039/C9SM00372J
16. Bosch FX, Ribes J, Díaz M, Cléries R (2004) Primary liver cancer: worldwide incidence and trends. Gastroenterology 127(5):S5–S16. https://doi.org/10.1053/j.gastro.2004.09.011
17. Casas-Grajales S (2015) Antioxidants in liver health. World J Gastrointest Pharmacol Ther 6(3):59. https://doi.org/10.4292/wjgpt.v6.i3.59
18. Chadha R, Bhandari S (2014) Drug–excipient compatibility screening—role of thermoanalytical and spectroscopic techniques. J Pharm Biomed Anal 87:82–97. https://doi.org/10.1016/j.jpba.2013.06.016
19. Chen H, Zhong Q (2015) A novel method of preparing stable zein nanoparticle dispersions for encapsulation of peppermint oil. Food Hydrocoll 43:593–602. https://doi.org/10.1016/j.foodhyd.2014.07.018
20. Dikpati A, Mohammadi F, Greffard K, Quéant C, Arnaud P, Bastiat G, Rudkowska I, Bertrand N (2020) Residual solvents in nanomedicine and lipid-based drug delivery systems: a case study to better understand processes. Pharm Res 37(8):149. https://doi.org/10.1007/s11095-020-02877-x
21. El-Helaly SN, Amr KA, Fadel M, Fahmy RH (2024) Fluticasone propionate zein nanoparticles—loaded in situ gelling system: in vitro/ex vivo studies and associated in vivo nasal MMP9 suppressed effect. J Drug Deliv Sci Technol. https://doi.org/10.1016/j.jddst.2024.105674
22. Elfalleh W, Tlili N, Nasri N, Yahia Y, Hannachi H, Chaira N, Ying M, Ferchichi A (2011) Antioxidant capacities of phenolic compounds and tocopherols from Tunisian pomegranate (*Punica granatum*) fruits. J Food Sci 76(5):C707–C713. https://doi.org/10.1111/j.1750-3841.2011.02179.x
23. Fang J-Y, Yu S-Y, Wu P-C, Huang Y-B, Tsai Y-H (2001) In vitro skin permeation of estradiol from various proniosome formulations. Int J Pharm 215(1–2):91–99. https://doi.org/10.1016/S0378-5173(00)00669-4
24. Fernández-Carneado J, Kogan MJ, Castel S, Giralt E (2004) Potential peptide carriers: amphipathic proline-rich peptides derived from the N-terminal domain of γ-zein. Angew Chem Int Ed 43(14):1811–1814. https://doi.org/10.1002/anie.200352540
25. Gordaliza M (2007) Natural products as leads to anticancer drugs. Clin Transl Oncol 9(12):767–776. https://doi.org/10.1007/s12094-007-0138-9
26. Hassan EA, Hathout RM, Gad HA, Sammour OA (2022) A holistic review on zein nanoparticles and their use in phytochemicals delivery. J Drug Deliv Sci Technol 73:103460. https://doi.org/10.1016/j.jddst.2022.103460
27. Hu K, McClements DJ (2014) Fabrication of surfactant-stabilized zein nanoparticles: a pH modulated antisolvent precipitation method. Food Res Int 64:329–335. https://doi.org/10.1016/j.foodres.2014.07.004
28. Ismail S, Garhy D, Ibrahim HK (2023) Optimization of topical curcumin spanlastics for melanoma treatment. Pharm Dev Technol 28(5):425–439. https://doi.org/10.1080/10837450.2023.2204926
29. Jurenka JS (2008) Therapeutic applications of pomegranate (*Punica granatum* L.): a review. Altern Med Rev J Clin Ther 13(2):128–144
30. Kalantari S, Roufegarinejad L, Pirsa S, Gharekhani M, Tabibiazar M (2021) β-Cyclodextrin-assisted extraction of phenolic compounds from pomegranate (*Punica granatum* L.) peel: a new strategy for anthocyanin copigmentation. LWT 151(March):112136. https://doi.org/10.1016/j.lwt.2021.112136
31. Kassem AA, Mohsen AM, Ahmed RS, Essam TM (2016) Self-nanoemulsifying drug delivery system (SNEDDS) with enhanced solubilization of nystatin for treatment of oral candidiasis: design, optimization, in vitro and in vivo evaluation. J Mol Liq 218:219–232. https://doi.org/10.1016/j.molliq.2016.02.081
32. Khattab A, Abouhussein DMN, Mohammad FE (2019) Development of injectable tenoxicam in situ forming microparticles based on sesame oil and poly-DL-lactide: characterization, efficacy and acute toxicity. J Drug Deliv Sci Technol 51:682–694. https://doi.org/10.1016/j.jddst.2019.04.001
33. Lai ECH, Lau WY (2005) The continuing challenge of hepatic cancer in Asia. Surgeon 3(3):210–215. https://doi.org/10.1016/S1479-666X(05)80043-5
34. Lai LF, Guo HX (2011) Preparation of new 5-fluorouracil-loaded zein nanoparticles for liver targeting. Int J Pharm 404(1–2):317–323. https://doi.org/10.1016/j.ijpharm.2010.11.025
35. Li H, Zhang X, Zhao C, Zhang H, Chi Y, Wang L, Zhang H, Bai S, Zhang X (2022) Entrapment of curcumin in soy protein isolate using the pH-driven method: nanoencapsulation and formation mechanism. LWT 153:112480. https://doi.org/10.1016/j.lwt.2021.112480
36. Mahmoud DB, Bakr MM, Al-karmalawy AA, Moatasim Y, El Taweel A, Mostafa A (2022) Scrutinizing the feasibility of nonionic surfactants to form isotropic bicelles of curcumin: a potential antiviral candidate against COVID-19. AAPS PharmSciTech 23(1):44. https://doi.org/10.1208/s12249-021-02197-2
37. Mahmoud DB, Wölk C, Schulz-Siegmund M (2023) Fabrication of 3D printed, core-and-shell implants as controlled release systems for local siRNA delivery. Adv Healthc Mater 12(31):1–16. https://doi.org/10.1002/adhm.202301643
38. Malviya S, Arvind Jha A, Hettiarachchy N (2014) Antioxidant and antibacterial potential of pomegranate peel extracts. J Food Sci Technol 51(12):4132–4137. https://doi.org/10.1007/s13197-013-0956-4
39. Motawi TK, El-Maraghy SA, ElMeshad AN, Nady OM, Hammam OA (2017) Cromolyn chitosan nanoparticles as a novel protective approach for colorectal cancer. Chem Biol Interact 275:1–12. https://doi.org/10.1016/j.cbi.2017.07.013
40. Mwamatope B, Tembo D, Chikowe I, Kampira E, Nyirenda C (2020) Total phenolic contents and antioxidant activity of *Senna singueana*, *Melia azedarach*, *Moringa oleifera* and *Lannea discolor* herbal plants. Sci Afr 9:e00481. https://doi.org/10.1016/j.sciaf.2020.e00481
41. Nakamura Y, Mochida A, Choyke PL, Kobayashi H (2016) Nanodrug delivery: Is the enhanced permeability and retention effect sufficient for curing cancer? Bioconjug Chem 27(10):2225–2238. https://doi.org/10.1021/acs.bioconjchem.6b00437
42. Nakhla DS, Mekkawy AI, Naguib YW, Silva AD, Gao D, Ah Kim J, Alhaj-Suliman SO, Acri TM, Kumar Patel K, Ernst S, Stoltz DA, Welsh MJ, Salem AK (2024) Injectable long-acting ivacaftor-loaded poly (lactide-*co*-glycolide) microparticle formulations for the treatment of cystic fibrosis: in vitro characterization and in vivo pharmacokinetics in mice. Int J Pharm 650:123693. https://doi.org/10.1016/j.ijpharm.2023.123693
43. Pan Z, Qu W, Ma H, Atungulu GG, McHugh TH (2012) Continuous and pulsed ultrasound-assisted extractions of antioxidants from pomegranate peel. Ultrason Sonochem 19(2):365–372. https://doi.org/10.1016/j.ultsonch.2011.05.015
44. Podaralla S, Perumal O (2012) Influence of formulation factors on the preparation of zein nanoparticles. AAPS PharmSciTech 13(3):919–927. https://doi.org/10.1208/s12249-012-9816-1
45. Radwan SAA, El-Maadawy WH, Yousry C, Elmeshad AN, Shoukri RA (2020) Zein/phospholipid composite nanoparticles for successful delivery of gallic acid into ahscs: influence of size, surface charge, and vitamin a coupling. Int J Nanomed 15:7995–8018. https://doi.org/10.2147/IJN.S270242
46. Saindane NS, Pagar KP, Vavia PR (2013) Nanosuspension based in situ gelling nasal spray of carvedilol: development, in vitro and in vivo characterization. AAPS PharmSciTech 14(1):189–199. https://doi.org/10.1208/s12249-012-9896-y
47. Sezgin-Bayindir Z, Yuksel N (2012) Investigation of formulation variables and excipient interaction on the production of niosomes. AAPS PharmSciTech 13(3):826–835. https://doi.org/10.1208/s12249-012-9805-4
48. Shirode AB, Bharali DJ, Nallanthighal S, Coon JK, Mousa SA, Reliene R (2015) Nanoencapsulation of pomegranate bioactive compounds for breast cancer chemoprevention. Int J Nanomed 10:475–484. https://doi.org/10.2147/IJN.S65145

49. Soltanzadeh M, Peighambardoust SH, Ghanbarzadeh B, Mohammadi M, Lorenzo JM (2021) Chitosan nanoparticles as a promising nanomaterial for encapsulation of pomegranate (*Punica granatum* L.) peel extract as a natural source of antioxidants. Nanomaterials 11(6):1439. https://doi.org/10.3390/nano11061439
50. Sreekumar S, Sithul H, Muraleedharan P, Azeez JM, Sreeharshan S (2014) Pomegranate fruit as a rich source of biologically active compounds. Biomed Res Int 2014:1–12. https://doi.org/10.1155/2014/686921
51. Surendhiran D, Li C, Cui H, Lin L (2020) Fabrication of high stability active nanofibers encapsulated with pomegranate peel extract using chitosan/PEO for meat preservation. Food Packag Shelf Life 23:100439. https://doi.org/10.1016/j.fpsl.2019.100439
52. Teaima MH, Badawi NM, Attia DA, El-Nabarawi MA, Elmazar MM, Mousa SA (2022) Efficacy of pomegranate extract loaded solid lipid nanoparticles transdermal emulgel against Ehrlich ascites carcinoma. Nanomed Nanotechnol Biol Med 39:102466. https://doi.org/10.1016/j.nano.2021.102466
53. Turrini E, Ferruzzi L, Fimognari C (2015) Potential effects of pomegranate polyphenols in cancer prevention and therapy. Oxid Med Cell Longev 2015:938475. https://doi.org/10.1155/2015/938475
54. van Meerloo J, Kaspers GJL, Cloos J (2011) Cell Sensitivity assays: the MTT assay. In: Methods in molecular biology, pp 237–245. https://doi.org/10.1007/978-1-61779-080-5_20
55. Viuda-Martos M, Fernández-López J, Pérez-Álvarez JA (2010) Pomegranate and its many functional components as related to human health: a review. Compr Rev Food Sci Food Saf 9(6):635–654. https://doi.org/10.1111/j.1541-4337.2010.00131.x
56. Yekdane N, Goli SAH (2019) Effect of pomegranate juice on characteristics and oxidative stability of microencapsulated pomegranate seed oil using spray drying. Food Bioprocess Technol 12(9):1614–1625. https://doi.org/10.1007/s11947-019-02325-8

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