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ORIGINAL PAPER

Nanoparticles of Punicalagin Synthesized from Pomegranate (*Punica Granatum L.*) with Enhanced Efficacy Against Human Hepatic Carcinoma Cells

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Abstract
Punicalagin is the most popular ellagitannin found in pomegranate husk and is well known to reduce the risk of cancer and cardiovascular diseases (CVDs). The present work describes a novel method for the preparation of nanoparticles of Punicalagin using pomegranate (*Punica granatum*) since the use of Punicalagin in bulk form is associated with many problems. In this study, nanoparticles of Punicalagin were synthesized, characterized using various techniques, and further evaluated for their antioxidant, antibacterial, and antiproliferative potential using HepG2 cancer cells. DLS analysis revealed that the average size of nanoparticles of Punicalagin was 87 nm, whereas SEM analysis confirmed spherical shaped nanoparticles with size ranging from 90 to 116 nm. HPLC studies confirmed the presence of Punicalagin in synthesized nanoparticles. The results also revealed that nanoparticles of Punicalagin were nearly four times more potent antioxidants than bulk and possessed an inhibition zone of about 13 mm. The response of antiproliferative assay showed that the nanoparticles of Punicalagin caused nearly 44% reduction while bulk form showed only a 15% reduction in cell viability of cancerous cells at 100 µg/mL. The study suggests a great potential for use of the herbal drug in nano form to treat cancer as compared to its bulk counterparts.

Keywords Punicalagin nanoparticles (PCN) · *Punica granatum* · Antibacterial · Antioxidant · HepG2 cancer cells · In-vitro antiproliferative activity

Introduction

Traditional and herbal medicine are having a strong impact on human life. Employing herbal medicines for the prevention of diseases is a very fruitful way with very little or no toxicity. This reinforces the scientists for the isolation and extraction of pharmacologically active compounds found in them. In the current study, *P. granatum* L., a shrub that belongs to the family *Lythraceae*, and a well-known source of various bioactive phytochemicals like tannins and phenolic compounds have been used. Pomegranate husk polyphenols extract exhibit various physiological properties which include their anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, and vasodilatory effects and also exhibit potent antioxidant activity [1]. Pomegranate husk extract can be used to target several diseases like cancer, diabetes, aging, cardiovascular disorder, and AIDS [2]. Various ellagitannins are present in pomegranate, but Punicalagin, accountable for the enhanced antioxidant potential of pomegranate husk is the major of all [3]. In nature, it exists as two reversible α- and β-anomers and is known as a potent antioxidant because of its ability to undergo hydrolysis in-vivo and in-vitro [2, 4].

Despite the stated benefits of ellagitannins, low absorption, poor bioavailability and less retention time may curtail their full potential. For example, under in vivo conditions, Punicalagin gets converted into ellagic acid and gets accumulated in epithelial cells of the intestine with poor absorption into the bloodstream. As a result, lower levels of ellagic acid are found in human blood even after

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the uptake of pomegranate juice. So, the commercially available Punicalagin has various side effects associated with it. In the direction to overcome the problems associated with Punicalagin, various approaches were followed by researchers such as the encapsulation of ellagitannins into biocompatible nanoparticles, these approaches are being exploited in cancer prevention [5–8]. However, all these methods use nanoparticles for encapsulation, which possesses the risk of toxicity and safety. Presumably, there is no published report of directly converting the Punicalagin into nano form without any encapsulation to date. We for the first time used the nano-technological approach to directly convert Punicalagin to its nano form using a similar approach as of our recently published articles [9, 10].

The present study deals with the synthesis of nanoparticles of Punicalagin using the extract of pomegranate husk. Synthesis of nanoparticles was followed by their characterization, evaluation of their antioxidant, antibacterial potential, and its comparison with that of bulk as well as with standard Punicalagin. The antiproliferative potential of synthesized nanoparticles of Punicalagin was also studied on HepG2 (hepatocellular carcinoma) cells.

Materials and Methods

Chemicals and Reagents

Punicalagins standard (a mixture of α- and β-Punicalagin, CAS No. 65995–63-3, ≥ 98% HPLC purity) was obtained from Sigma Aldrich. Phosphate Buffer Saline (PBS), methanol, ethanol, phosphoric acid, HPLC grade water, acetonitrile, glacial acetic acid, and nutrient agar were purchased from Fisher Scientific, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was bought from SRL, fetal bovine serum, antibiotic mix, Dulbecco's Modified Eagle Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and nutrient broth were procured from HiMedia. Trypan blue was procured from CISCO. All chemicals and reagents used were of analytical grade.

Cell Culture and Cell Lines

The microbial strain of *Escherichia coli* (DH5α) was procured from one of the Govt. hospitals of New Delhi. The human hepatocellular carcinoma cells (HepG2) were obtained from National Centre for Cell Sciences, Pune.

Instruments and Equipment

A scientific sieve of standard size 4.75 mm to filter pomegranate powder was used. Ultrasonicate Bath, TPC-25, from Roop Telesonic Ultrasonics, was used to process the bulk form. Dynamic Light Scattering (Nano plus, DLS, Malvern Zetasizer S90 series) was used to analyze the particle size of synthesized nanoparticles, Centrifuge (R-24) from Remi, Lyophilizer, Light Microscope, Electronic balance (BT-124-S) from Sartorius Stedim India Pvt. Ltd., New Delhi, India. Magnetic Stirrer (1MLH) from Remi was used to agitate the extract. Biogene Incubator shaker, Biotechnologies Inc., Delhi, India, and Autoclave, Reliance Instruments Corporation was used for incubation and sterilization of chemicals, respectively. Rotavapor (R-210) was used to concentrate the sample. The surface morphology of synthesized nanoparticles was studied using a Scanning Electron Microscope (EVO-18, Carl Zeiss, Germany). Deep Freezer − 20 °C (Blue star), UV Visible Spectrophotometer (Spectro UV–Vis Dual beam and Auto Cell UVS 2700, Labomed, INC, Germany), 5% CO_2 Incubator (MCO-5 M MCO multi-gas incubator) and ELISA Reader (Thermofisher Scientific) were used for in-vitro applications. Fourier Transformed Infrared Spectroscopy (FTIR) and High Permissible Liquid Chromatography (HPLC) 1100 from Agilent Technologies were used to carry out characterization. Cell morphology was studied using an Inverted Tissue Culture Microscope (LEICA DMI8, Leica Microsystem, France).

Collection and Pretreatment of Pomegranate

Sample preparation process including manual size reduction (knife cutting), drying and milling, and sieving of pomegranate. Fresh fruits of *P. granatum* were collected from the fruit market of Delhi, India, and separated into husk (pericarp), peel (membranes/mesocarp), and arils (containing the seeds). Fruit pericarp of pomegranate was broken into smaller pieces to aid drying under sunlight for 3–4 days and further ground with the help of a mixer grinder. The fine powder was separated through sieving and used throughout the study [11].

Extraction of Punicalagin and Synthesis of Nanoparticles from Pre-treated Pomegranate

The extraction of Punicalagin from pomegranate husk was conducted using the method of Lu et al. with modifications [12]. One gram of dried powder was extracted ultrasonically in 30 mL ethanol (40%) for 30 min, the process was repeated twice to obtain the bulk form of extract (PCB). The obtained bulk was centrifuged at 12,000×*g* for 2 min at 30 °C. The supernatant was combined each time after centrifugation and the ethanol was allowed to vaporize using the rota vaporization technique. The concentrated sample was lyophilized at − 40 °C to obtain a dry lyophilized powder of the same. Thereafter, 30 mg of the

above powdered was dissolved in a 10 mL of methanol–water (1:1) filtered through a 0.22 µm membrane filter to obtain nanoparticles of Punicalagin (PCN). The working solution of the Punicalagin standard (PCS) was also prepared in methanol (1 mg/mL).

Characterization Studies

The isolated bioactive compound was characterized first using UV-Visible Spectrophotometer followed by Particle Size Analyzer (DLS), Scanning Electron Microscope (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and High Permissible Liquid Chromatography (HPLC).

UV Visible Spectroscopy

Spectrum analysis using a UV visible spectrophotometer, the primary method to get confirmation of isolated isomers of the bioactive compound, Punicalagin was carried out to compare the spectrum of PCN with PCS and PCB by comparing their lambda max. The spectra of all three samples were analyzed.

Estimation of Particle Size (DLS)

Particle size Analyzer assisted us to understand the physical characteristics of the sample. Dynamic light scattering (DLS) measures fluctuations in the intensity of light due to particle Brownian motion. It is a reliable technique for accurate estimation of average particle size. PCN was subjected to DLS for the estimation of average particle size [13, 14].

Scanning Electron Microscope (SEM)

Direct visualization of nanoparticles is possible using this electron microscopy. Size as well as the shape of synthesized nanoparticles can be determined using SEM. SEM was carried out to visualize the surface morphology of PCN. The sample was staged on a sample holder followed by gold coating to make it conductive. The analysis was done by scanning the sample with a sharp beam of electrons. Emitted electrons from the surface of the sample help to determine its surface characteristics [15].

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy is a flexible way to evaluate the functional groups present in a sample because various functional groups absorb particular IR radiation frequencies. Every molecule also has a special spectrum known as a fingerprint which can quickly distinguish one molecule from another. The infrared region has been categorized into further three sub-regions i.e. near IR region (overtone region), mid-IR region (vibration rotation region), and far IR region (rotation region) [16].

The bands occurring can be defined according to the characteristics of the functional groups present in the compound. Nanoparticles have a high surface-to-volume ratio that makes them show distinctive characteristics [17].

High Permissible Liquid Chromatography (HPLC)

For HPLC analysis of Punicalagin from herbal source pomegranate, mentioned chromatographic conditions were applied. Kromasil 100-3.5-C-18 (4.6 × 100 mm) column having column temperature of 30 °C, λ 260 nm, isocratic pump (105 psi), and VWD detection system was used. The flow rate maintained was 0.8 mL/min. All solutions were filtered through 0.22 µm pore cellulose membrane and 10 µL volume was injected [18].

Mobile Phase Preparation

The mobile phase for the separation of isomers of Punicalagin, consisted of acetonitrile and glacial acetic acid (2%, v/v) in a ratio of 20:80 (v/v), prepared in HPLC grade water to carry out HPLC analysis of the samples after degassing of the solvents.

Standard Stock Solution

Punicalagin procured from sigma was dissolved in methanol (1 mg/ml) to be used as a standard to compare samples. The chromatogram was used as a reference.

Sample Preparation

Nanoparticles synthesized from pomegranate husk were injected in HPLC injector to record chromatograms and compared with the chromatogram of standard.

Calculation Involved

Concentration of Punicalagin in nanoparticles sample was calculated by using the formula:

$$\text{Concentration of sample} = [\text{Area sample}/\text{Area Standard}] * \text{concentration of Standard}$$

Determination of Antibacterial Activity

Agar well diffusion method was used to evaluate the antimicrobial activity of PCN against gram-negative bacterial (*E. coli*). The protocol by Balouiri and Dahham with

their co-researchers was used with slight modifications [19, 20]. Agar plates with the known concentration of *E.-coli* were prepared followed by punting a well (hole) with a diameter of 5–6 mm aseptically with a sterile tip. The bacterial concentration used was standardized to 1×10^6 CFU/mL and 0.1 mL of bacterial culture was spread on each agar plate. Furthermore, 20 µL of the prepared nanoparticles were added into each well of the test plate. Control plate having bacteria but no PCN and test plate with bacterial culture and PCN were incubated for 16–18 h at 37 °C. PCS and PCB were also added individually in the same volume as that of PCN. The concentration of all the samples was 1 µg/mL. The combined mixture of methanol and water (1:1 v/v) was used as a solvent to carry out this experiment. As soon as the incubation period was over, the mean diameter (mm) of the inhibition zone formed on the test plate was measured from the margin of the zone to the brink of the well.

Determination of Antioxidant Activity

The DPPH assay was done to evaluate antioxidant activity. This assay measures the free radical scavenging capacity of the investigated extract. In the presence of an antioxidant, which can provide an electron to DPPH (contains stable free radical), the purple color of free DPPH radical vanishes, and the change in absorbance is measured at $\lambda = 517$ nm [21].

DPPH solution of 0.1 mM strength was prepared in methanol and 2 mL of this solution was added to 1 mL of PCB, PCN, and PCS each. After incubation of 30 min at room temperature, the absorbance of the solution was recorded by using an ultraviolet (UV) spectrophotometer at 517 nm and DPPH was taken as control. The experiment was performed in triplicates. The inhibition of the DPPH radical by the sample was calculated as per the formula:

$$\%Inhibition = \frac{(Acontrol - Asample)}{Acontrol} \times 100$$

where $A_{control}$ is the absorbance value of the control reaction and A_{sample} is the absorbance value of the sample [22].

In-Vitro Studies

Nanoparticles synthesized from pomegranate husk were subjected to determine their in-vitro antiproliferative potential using the HepG2 cells.

The protocol to determine the antiproliferative potential of PCN is mentioned below.

Maintenance of Cell Culture and Cell Seeding

DMEM was used to culture the HepG2 cells. The medium was supplemented with 10% FBS and 1% antibiotic mix in a T-25 flask and incubated at 37 °C using 5% CO_2 in a humidified CO_2 chamber. The viability of cells was determined by staining cells with trypan blue and counting using a hemocytometer under a microscope. Cells were seeded keeping a cell density of 20,000 cells/mL and from this cell suspension, 100 µL was pipetted into each well of a 96-well plate and incubated for 24 h in a 5% CO_2 incubator at 37 °C to carry out MTT assay [23].

Assessment of Cell Proliferation

Analysis of the antiproliferative potential of PCN was done using MTT assay based on the method described by Badawi and co-workers with slight modifications [5]. Different concentrations of PCN and PCB (20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL) were individually added in separate wells (in triplicates) of a 96-well plate.

Plates were kept at 37 °C in an atmosphere of 95% air and 5% CO_2 for 48 h followed by the addition of 20 µL of MTT reagent (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 0.5 mg/mL PBS) in each well keeping them undisturbed for next 4 h at the same temperature. Subsequently, 50 µL of DMSO was added to each well, and plates were kept for incubation for another 15 min to dissolve blue crystals and the absorbance was recorded by a microplate reader at a wavelength of 570 nm. The relative change in percent viability of cells treated with various concentrations of the synthesized nanoparticles was calculated for untreated control having 100% cell viability.

The percent cytotoxicity was determined [24] using the following formula:

$$\%\ cytotoxicity = \frac{(Optical\ density\ of\ sample)}{(Optical\ density\ of\ control)} \times 100 \quad (1)$$

Morphological Studies

The HepG2 cancer cells were cultured using coverslips with a density of 10^3 cells/coverslip. The sample was added to the cells. After 48 h of incubation, sample coverslips were carefully mounted on clean slides and were subjected to the analysis of morphological changes. The microscope was used to study cell morphology at 40 × magnification. Morphology of cells before treatment with PCN synthesized from pomegranate husk extract was also studied.

Results and Discussion

Nanoparticles synthesized from pomegranate husk extract were subjected to various characterization techniques.

Characterization Studies

Results of different characterization tools used to study nanoparticles synthesized from pomegranate husk extract are discussed.

UV Visible Spectroscopy Analysis

The primary confirmation study was done using a UV Visible spectrophotometer which revealed the presence of α and β isomers of Punicalagin in PCN at 254 nm and 378 nm, respectively. PCS also showed peaks at the same lambda max, confirming the successful extraction and preparation of nanoparticles of Punicalagin. PCB did not show any differentiated peaks of Punicalagin due to the presence of various phytochemicals in it (Fig. 1).

Dynamic Light Scattering (DLS)

The results of the particle size analyzer showed that an average particle size of PCN was about 87 nm (Fig. 2), whereas PCB has shown an average particle size of about 2 µm. It has been observed that the nanoformulation was about twenty to twenty-five times smaller in size as compared to bulk extract.

Scanning Electron Microscopy (SEM)

The SEM images showed PCN were spherical with well-dispersed particles ranging from 90 to 116 nm in size (Fig. 3b). While the SEM image of PCB showed irregular morphology with micro-size particles having murkiness all around (Fig. 3a).

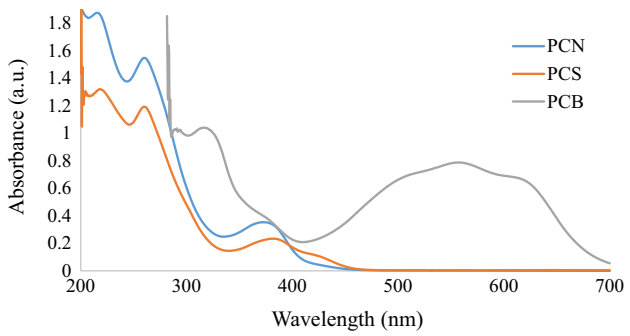


Fig. 1 UV–visible spectra of PCS, PCN, and PCB

Fourier Transform Infrared Spectroscopy (FTIR)

For PCN, isolated from the husk of pomegranate extract, the spectrum was scanned over a wavenumber range of 4000–500 cm^{-1}. PCS was also subjected to FTIR. PCN (Fig. 4b) had shown significant changes in the FTIR as compared to standard (Fig. 4a). At higher wavenumbers, the FTIR spectrum of the PCS exhibited broadband at 3336 cm^{-1} corresponded to the strong intramolecular hydrogen bond (OH). Two quietly broad bands were observed at 1738 and 655 cm^{-1} which have the stretching vibrations of the δ(C = N), ν(C=C=C), and ν(C–H).

The broad peak at 3300 cm^{-1} and 1643 cm^{-1} indicated the change in O–H and N–H stretching in PCN and indicated that the OH group was also involved in hydrogen bonding. The disappearance of bands in PCN at 1738 cm^{-1} indicated the breaking of C=C=C bonds. The changes in the FTIR confirm the similarity between PCS and PCN synthesized from pomegranate husk extract.

High Permissible Liquid Chromatography (HPLC)

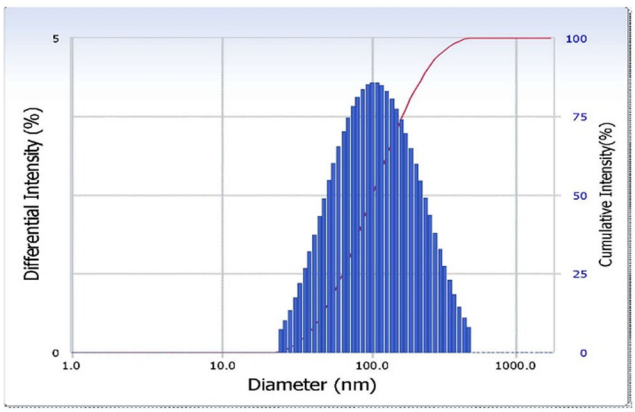
The isolation of Punicalagin from herbal source, pomegranate husk was confirmed by elution of α and β isomers of Punicalagin at 1.2 min and 1.5 min retention time (t_R), respectively which was found similar to PCS obtained from Sigma Aldrich. The two isomers in both PCS (Fig. 5a) and PCN (Fig. 5b) showed similarity in elution of peaks which was the strong justification for successful isolation and preparation of PCN. The yield of isomers of Punicalagin in the sample was also calculated and it was found to be 1100 ng/ml.

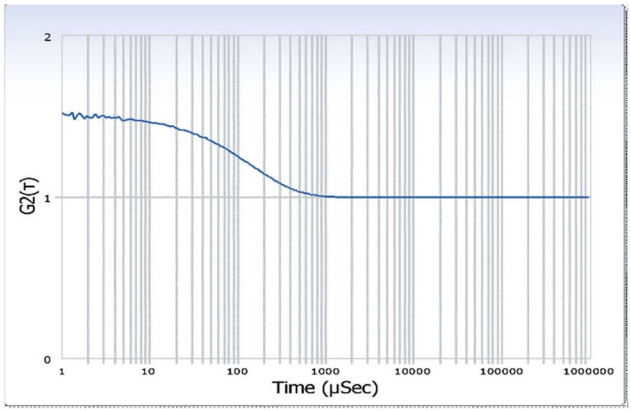
Antibacterial Activity

Test plate with PCN and bacterial culture produced inhibition zone of average size 13 mm for *E. coli* (Fig. 6b) and control plate with bacteria but no nanoparticles in it was also observed after incubation time ended (Fig. 6a). PCS showed a zone of inhibition of 12.2 mm (Fig. 6d), whereas PCB did not show any antibacterial potential (Fig. 6c). Wasim and co-workers also reported similar results with methanolic extract of *P. granatum* fruit husk to be active against *E. coli* [25]. It showed that extract obtained from pomegranate husk was active and effective against the micro-organism.

Antioxidant Activity

The results of antioxidant property using DPPH scavenging activity revealed that bulk form of pomegranate husk extract was not as effective as PCN showing approximately





Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	133.8	87.2
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	133.8	87.2
Residual :	3.067e-003	(O.K)

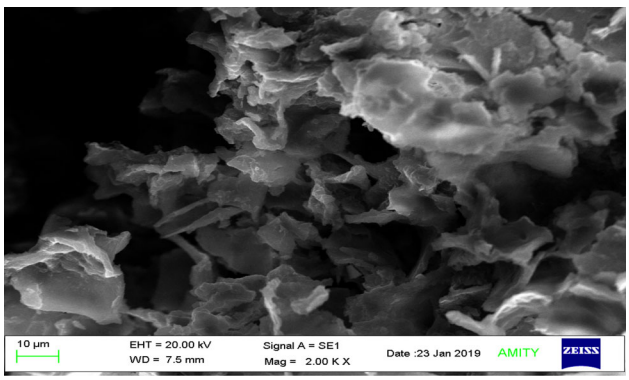
Cumulants Results

Diameter	(d)	: 87.4	(nm)
Polydispersity Index	(P.I.)	: 0.282	
Diffusion Const.	(D)	: 5.640e-008	(cm²/sec)

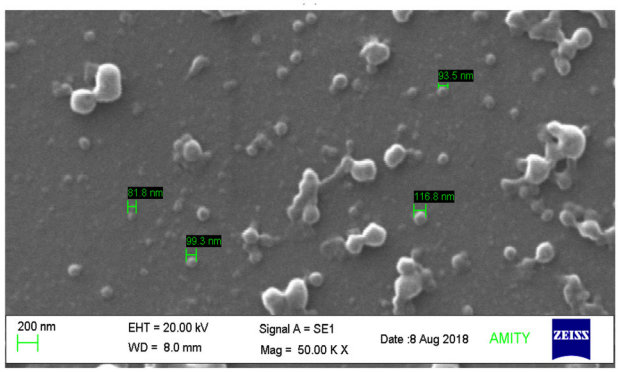
Measurement Condition

Temperature	: 25.1	(°C)
Diluent Name	: WATER	
Refractive Index	: 1.3328	
Viscosity	: 0.8858	(cP)
Scattering Intensity	: 28529	(cps)
Attenuator 1	: 7.28	(%)

Fig. 2 DLS image of PCN



(a)



(b)

Fig. 3 SEM images of **a** bulk extract of Punicalagin (PCB) and **b** synthesized nanoparticles of Punicalagin (PCN)

four times more antioxidant activity (inhibition 82%) than PCB (inhibition 22%). PCS showed 85% inhibition (Fig. 7). The antioxidant activity in nano form enhanced, this might be due to interference of various other phyto-components present in bulk. Our results showed that inhibition of DPPH radical by PCN was more as compared to PCB. This might be due to pomegranate polyphenols which are the main component capable of restraining the effect of ROS on the body as already reported by Akram and co-researchers [26]. Similar results were also reported by us in our earlier studies using nano-stains synthesized from mushroom as the conversion of herbal extract into nano form makes it more effective in scavenging DPPH radicals due to its higher potential for antioxidant activity than their respective bulk extracts [10, 27].

Antiproliferative Studies

The cell viability of cancerous cells got tailed off significantly in a dose-dependent manner concerning the untreated control group on treatment with PCN. There was a 4% reduction in cancerous cells at a lower concentration (20 μg/mL) of nanoparticles. A significant decrease in percent viability was observed as soon as the dose was increased to 40 μg/mL and 60 μg/mL. The fall-off shown was approximately 27% for untreated cells. Further, the cell viability of cancerous cells was decreased from 100% (untreated cells) to 62% (80 μg/mL) and further to 56%

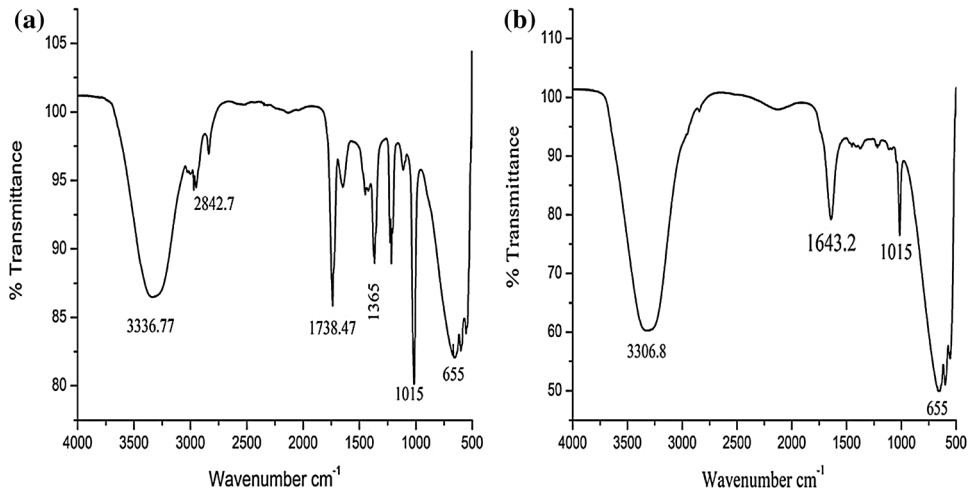


Fig. 4 FTIR spectra of **a** standard Punicalagin (PCS) and **b** synthesized nanoparticles of Punicalagin (PCN)

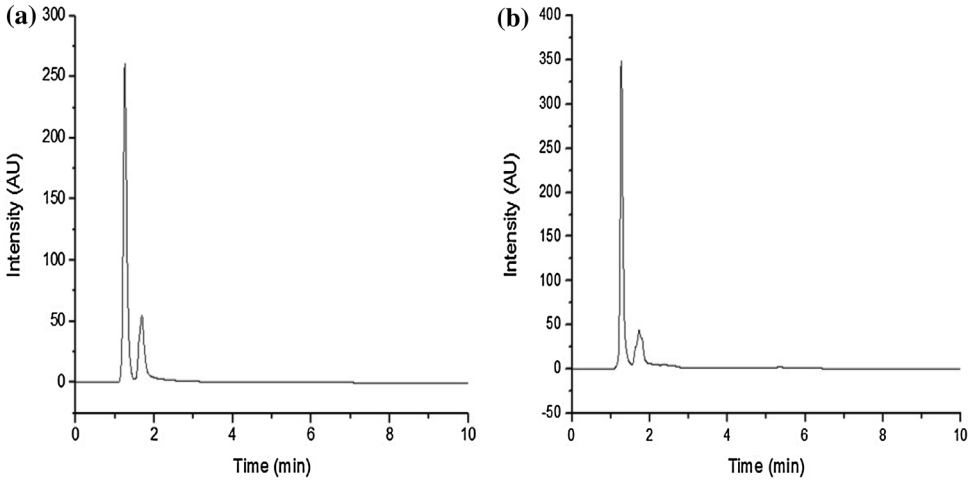


Fig. 5 HPLC chromatogram of **a** standard Punicalagin (PCS), **b** synthesized nanoparticles of Punicalagin (PCN)

(100 μg/mL) upon the addition of nanoparticles. On contrary to PCN, PCB did not show a significant decrease in cell viability of cancerous cells even at 100 μg/mL (85%) (Fig. 8). These results showed that nanoparticles synthesized from pomegranate husk have an inhibiting effect on the cancerous cells. This can be attributed to the presence of an active ingredient, Punicalagin in the herbal nano extract synthesized from pomegranate husk which has lessened the growth of cancer-causing cells by 4% even at lower concentration (20 μg/mL). With an increase in the concentration of nanoparticles, cell viability was observed to be reduced further. The dose-dependent effect of nanoparticles of Punicalagin was observed which was following the study reported by Panth et al. [28]. Another study on Raji lymphoma cells treated by *Cuscuta* extracts was done and these findings supported the fact that many natural plant extracts could inhibit proliferating cancer cells [29, 30].

The comparative results showing the differences in PCN and PCB are summarized in Table 1.

Morphological Studies

The morphology of cancerous cells treated with nanoparticles was also studied. It was observed that after the addition of PCN, the cell membrane started rupturing and

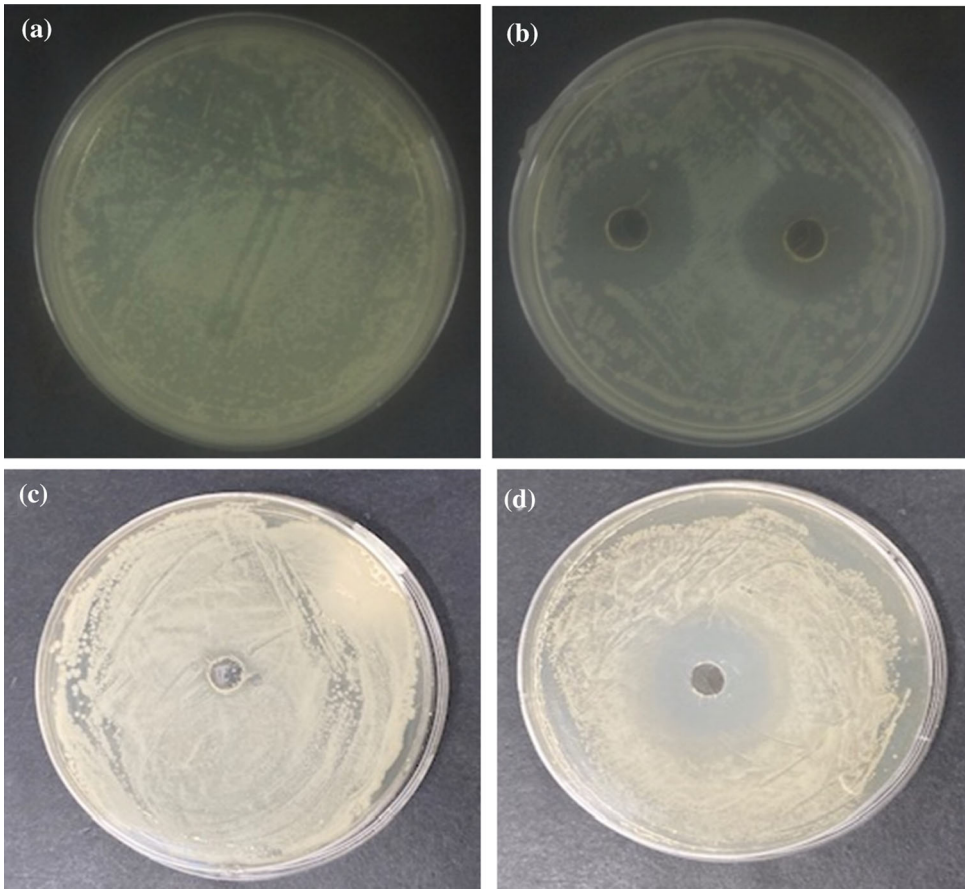


Fig. 6 Antibacterial activity of **a** control (without nanoparticles), **b** nanoparticles of Punicalagin (PCN), **c** bulk extract of Punicalagin (PCB), and **d** standard Punicalagin (PCS)

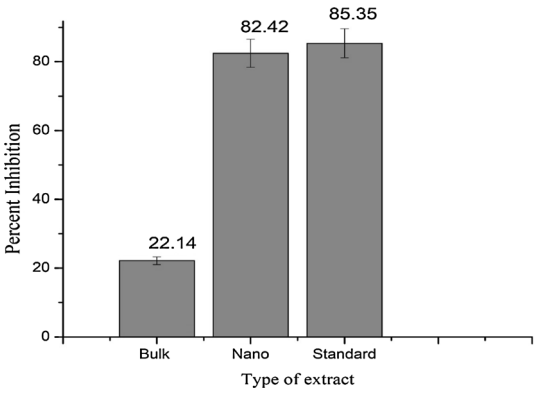


Fig. 7 Antioxidant activity of bulk extract of Punicalagin (PCB), nanoparticles of Punicalagin (PCN) and standard Punicalagin (PCS). Here, error bars represent standard error among three readings

the cellular components initiated coming out of the cell due to lysis of cancerous cells (Fig. 9b). Cell clumping and giant cells undergoing membrane disintegration were also seen as post-effects of the addition of PCN. On the contrary, the cancerous cells without nanoparticles showed round and spherical dominant cell boundaries with an intact nucleus and other cellular components. The monolayer was observed in cells before treatment (Fig. 9a). PCN rendered an increase of membrane rupture of the cells revealing their role in disrupting the structural integrity. Similar morphological changes, suggesting induction of apoptosis of various human tumor cells were also observed by Valter and co-workers while performing experiments of in-vitro screening of ethanol extracts prepared from various commercial plants. At extract concentration of 20 µg/mL, apoptosis (small aberrant nuclei, membrane blebbing, nuclear fragments) was induced in cells, whereas the untreated sample showed only round and uniform cells growing in a monolayer shape [31]. An increase in membrane disintegration by nanoparticles can be associated with the oxidative stress induced by most anticancer agents including Punicalagin leading to mitochondrial dysfunction [32].

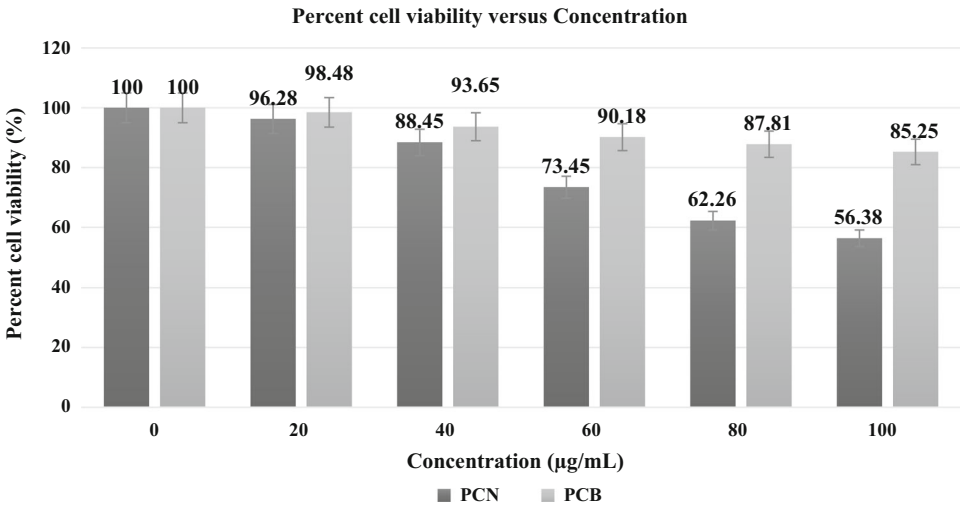


Fig. 8 Graph showing percent cell viability of HepG2 versus concentration of both nanoparticles of Punicalagin (PCN) and bulk extract of Punicalagin (PCB). Here, error bars represent standard error among three readings

Table 1 Comparison of a bulk extract of Punicalagin and nanoparticles of Punicalagin with various instrumental parameters

S. no	Parameter studied	Bulk extract of Punicalagin (PCB)	Nanoparticles of Punicalagin (PCN)
1	SEM	Irregular morphology with micro-size particles	Spherical morphology with particle size ranges from 90 to 116 nm
2	DLS	Average size: 2 Microns	Average size: 87 nm
3	Antioxidant activity	Inhibition offered: 22%	Inhibition offered: 82%
4	Antiproliferative potential	Cell viability: 85%	Cell viability: 56%

This study provided a new method for the preparation of nanoparticles of Punicalagin from pomegranate husk to overcome the side effects associated with bulk Punicalagin which is reported to have anti-inflammatory, anticancer, and anti-atherosclerotic properties.

However, individual compounds present in the extract of pomegranate need to be investigated in detail before drawing any conclusion. Some clues drawn from another study revealed that the pomegranate extracts (juice and oil) inhibited proliferation which leads to induced apoptosis in androgen-dependent and independent prostate cancer cell lines. Remarkably, pomegranate unable to cause cytotoxicity in normal prostate epithelial cells. These findings indicated that nanoparticles of Punicalagin possess antiproliferative activity which makes them nanoparticles of choice to treat cancer. The results of studies done on cancer cells were quite encouraging.

However, extensive studies to check bioavailability of the same are yet to be explored which will give us better insight for the application of synthesized nanoparticles of Punicalagin at the commercial level to treat cancer.

Conclusion

The study demonstrates a novel method for the preparation of nanosize Punicalagin from its herbal source without any need for encapsulation. The prepared nanoparticles were characterized and examined for their effects in the HepG2 cancer cell-line. The characterization studies showed prepared nanoparticles were in the size of 90–116 nm, well-dispersed with an average size of 87 nm. This is the first time that our study showed the superiority of nano-size Punicalagin in enhancing the inhibition of cell growth compared to their bulk counterpart and even much more beneficial compared to its various nano encapsulated formulations. Our method does not include or produce any synthetic or toxic nanoparticles. This implies the anticancer effect of pomegranate extract might be enhanced by nanoparticles of Punicalagin for alternative approaches in therapeutics. These results provide us insight for future research to evaluate the bioavailability and potential of the synthesized nanoparticles of Punicalagin by subjecting them to other cancer cells responsible for various other types of cancer and solid tumors.

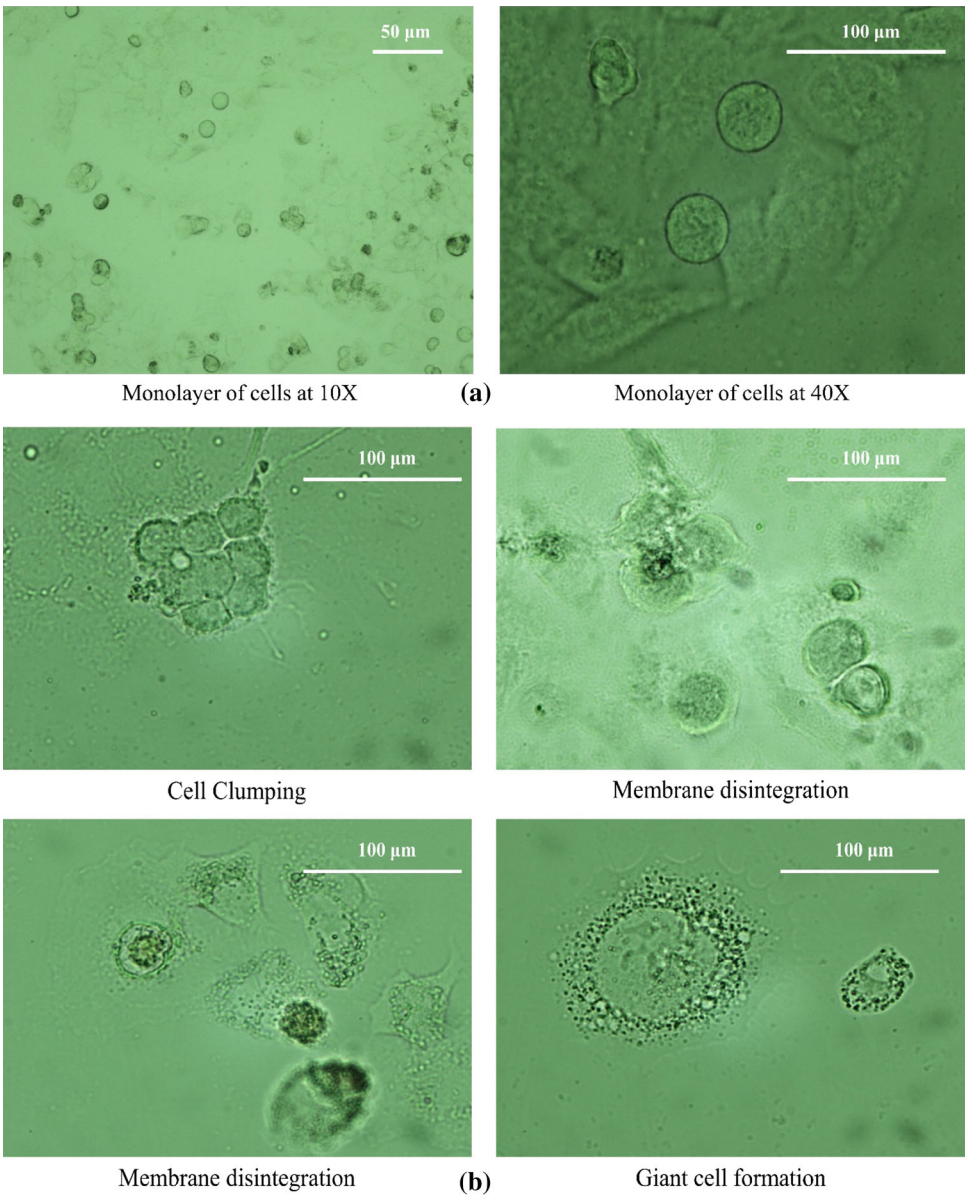


Fig. 9 Morphology of HepG2 cells using tissue culture microscope: **a** before the addition of nanoparticles of Punicalagin (PCN) and **b** after addition of nanoparticles of Punicalagin (PCN)

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Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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