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Punicalagin, a polyphenol from pomegranate fruit, induces growth inhibition and apoptosis in human PC-3 and LNCaP cells

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ABSTRACT

Prostate cancer (PCa) is an international health problem and search for its effective treatment is in progress. Punicalagin (PN), polyphenol from pomegranate fruit, is known to exhibit potent anticancer activity in lung, breast and cervical cells. However, there is paucity of information on its effect in PCa. This study evaluated anti-proliferative effects of PN and its effects on extrinsic pathway of apoptosis in PCa cells, and angiogenesis in chicken chorioallantoic membrane (CAM). Antioxidant activities of PN were determined by 2,2–diphenyl-1-picryhydrazyl (DPPH) radical scavenging and inhibition of lipid peroxidation (LPO) methods. PCa (PC-3 and LNCaP) and normal prostate (BPH-1) cells were cultured and treated with PN (10, 50 and 100 μM). Cytotoxicity and viability effects of PN were determined by lactate dehydrogenase (LDH) and XTT assays, respectively. Antiangiogenic effects were measured using CAM assay, while apoptosis was assessed by DNA fragmentation, enrichment factor by Cell Death Detection ELISA kit and expressions of caspases-3 and -8. Results showed that PN (10–200 μM) significantly scavenged DPPH and inhibited LPO in a concentration-dependent manner. Furthermore, PN (10–100 μM) concentration-dependently inhibited viability in PC-3 and LNCaP, while viability in BPH-1 was insignificantly affected. PN had low toxicity on cells *in vitro* at concentrations tested. Also, PN (100 μM) increased enrichment factor in PC-3 (2.34 ± 0.05) and LNCaP (2.31 ± 0.26) relative to control (1.00 ± 0.00). In addition, PN (50 μM) decreased the network of vessels in CAM, suggesting its anti-angiogenic effect. Moreso, PN increased the expressions of caspases-3 and -8 in PC-3. Overall, PN exerts anti-proliferative activity in PCa cells via induction of apoptosis and anti-angiogenic effect.

1. Introduction

Prostate cancer is a major health problem in the world. It is the most frequently diagnosed cancer among men and the second leading cause of male cancer deaths [6]. Although, there are many therapeutic strategies including chemotherapy, radiation and combination therapies, however, high systemic toxicity and drug resistance limit the successful outcomes in most cases [8]. Natural products and their synthetic derivatives may be considered as a potential source of novel compounds for the treatment of cancers. Indeed, over 60% of the approved anticancer drug candidates developed between 1981 and 2002 are derived from natural products [30]. According to the World Health Organization (WHO), 80% of the population in Africa and some Asian countries still use herbal preparations to treat their illnesses, including cancer [10]. Therefore, research exploring the medicinal usefulness of indigenous plants and natural products is important and relevant.

Pomegranates (*Punica granatum* Linn.) have been used for medicinal purposes against diarrheal, gum, parasitic and inflammatory disorders [22]. Pomegranate extracts have been shown to exhibit anticancer effects such as anti-proliferative, pro-apoptotic, anti-invasive, and/or anti-inflammatory properties in cancer cell lines [1,2,4,23,34,37]. Also, pomegranate extract has been reported to reduce the growth of human prostate and lung cancer xenografts in immune-deficient mice and suppressed prostate tumorigenesis in

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the TRAMP mouse model [3,24]. Pomegranates contain polyphenolic compounds with high antioxidant and free-radical-scavenging activity, including flavonoids, condensed tannins and hydrolyzable tannins (ellagitannins and gallotannins) [20] and [19]. Ellagitannins are the most bioactive polyphenols of pomegranates and the most abundant ellagitannin in pomegranates is punicalagin (PN) [19]. PN is responsible for more than half of the total antioxidant capacity of the pomegranates juice [14]. Anti-atherosclerotic and antioxidant properties of pomegranate have been linked to its high polyphenol content especially of PN [1,35]. PN has been shown to elicit remarkable biological activities including anti-inflammatory [5,38], hepatoprotective [26] and [27] and anti-genotoxic activities [40]. Despite the anti-cancer effect of PN reported in literature, there is still dearth of information on its effect in androgen-dependent and −independent PCa cells. Hence, this study was designed to evaluate the anti-proliferative effects of PN and its effects on extrinsic pathway of apoptosis in human PCa cells and, angiogenesis in chicken chorioallantoic membrane.

2. Materials and methods

2.1. Chemicals and reagents

Ethylenediamine tetra-acetic acid (EDTA), 2,2−diphenyl-1-picryhydrazyl (DPPH), 2- deoxyribose, Folin-Ciocalteu reagent, catechin, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), ascorbic acid and punicalagin were purchased from Sigma Chemical Co., Saint Louis, MO, USA. Ferrous ammonium sulphate, hydrochloric acid, naphthylenediamine dihydrochloride and sodium hydroxide were procured from British Drug House (BDH) Chemical Ltd., Poole, UK. Other chemicals were of analytical grade and purest quality available.

2.2. Cells

PC-3, LNCaP and BPH-1 cell lines were acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). For PC-3 and LNCaP, the culture medium consisted of RPMI 1640 medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum gold (PAA Laboratories, Pasching, Austria), penicillin (100 units/ml), and streptomycin (100 μg/ml) which was used for cell growth in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For BPH-1, the culture medium consisted of RPMI 1640 medium supplemented with 20% fetal bovine serum gold, dihydrotestosterone (20 ng/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), insulin (5 μg/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml).

2.3. Preparation of punicalagin

Prior to the experiments, 10 mg of PN (Sigma) was dissolved in 80 μl of DMSO and diluted to the desired concentrations with culture media to give a water-soluble fraction in which DMSO concentration did not exceed 0.02% in the highest of the concentrations applied.

2.4. Animals

Male Wistar rats weighing between 200-220 g were purchased from the central animal house, Department of Physiology, University of Ibadan, Nigeria. The animals were kept in well-ventilated cages at room temperature (28−30 °C) and maintained on laboratory chow (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum*. The rats were allowed a period of two weeks for acclimatization after delivery, and then used for lipid peroxidation assay. Experimental protocols, handling and treatment of rats were approved by the Animal Ethics Committee of Faculty of Basic Medical Sciences, University of Ibadan, Nigeria.

2.5. Preparation of rat liver homogenate

Rats were sacrificed under light ether anaesthesia and liver excised after dissection. Liver was removed and washed in ice-cold 1.15% KCl solution, dried and weighed. It was homogenized in 4 vol of 50 mM phosphate buffer, pH 7.4 and centrifuged at 10,000 × *g* for 15 min to obtain the post-mitochondrial fraction (PMF), which was used for lipid peroxidation assay.

2.6. Antioxidant assays

2.6.1. Determination of DPPH radical scavenging activity of PN

The stable DPPH radical was used for the determination of free radical scavenging activities of PN as described by Ref. [28]. A portion (1 ml) each of the different concentrations (10−200 μM) of PN was added to 1 ml of 1 mM DPPH in methanol. The mixtures were vortexed and incubated in a dark chamber for 30 min after which the absorbance was measured at 517 nm against a DPPH control containing only 1 ml of methanol in place of PN. The procedure above was repeated but PN was replaced with a standard antioxidant (Catechin).

All procedures were carried out in triplicates.

The inhibition of DPPH was calculated as a percentage using the expression:

$$\%I = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100$$

Where % I is the percentage inhibition of the DPPH radical, Acontrol is the absorbance of the control and Asample is the absorbance of the test compound.

2.6.2. Inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation by PN

Inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation was carried out by the method of [31]. The reaction mixtures contained 0.2 ml of rat liver homogenate in varying concentrations of 30 mM tris-buffer, 0.38 ml of 0.16 mM ferrous ammonium sulphate, 0.06 ml ascorbic acid and different concentrations of PN (10−200 μM) and, were incubated for 1 h at 37 °C. The resulting thiobarbituric acid reacting substances (TBARS) formed were measured as followed; briefly, an aliquot (0.4 ml) of the reaction mixture was mixed with 1.6 ml of 0.15 M Tris-KCl buffer and 0.5 ml of 30% TCA (to stop the reaction), and placed in a water bath for 45 min at 80 °C. After which it was cooled in ice and centrifuged at room temperature for 15 min at 3000 *g* to remove precipitates. The absorbance of the clear pink coloured supernatant was measured against blank at 532 nm. Catechin was used as standard and the experiment performed in triplicate.

2.7. Assays for cell viability, cytotoxicity and DNA fragmentation

2.7.1. Measurements of cell viability

PC-3, LNCaP and BPH-1 (1×10^4) cells were cultured in a 96-well microtiter plate containing 0.1 ml of RPMI growth media/well for 24 h. Cells were incubated with PN dissolved in dimethyl sulfoxide (DMSO)/media (DMSO less than 0.02%), and PN concentrations of 10, 50 and 100 μM were used with incubation period of 24−96 h. Cells were evaluated for their viability using the XTT assay (Roche Applied Science, Mannheim, Germany) as described by Refs. [13] and [33]. After incubation period, 50 μl of the XTT solution was added to the treated cells and incubated in dark for 4 h at 37 °C

under standard conditions of 5% CO_2 until the orange formazan product became visible. The absorbance was read on ELISA plate reader (Thermo Electron Corporation, Waltham, Massachusetts, USA) between 450-500 nm. The blank values (medium) were subtracted from each well of the untreated and treated cells. The results were reported as percentage of viable cells where the absorbance of untreated cells was set as 100%.

2.7.2. Lactate dehydrogenase (LDH) assay

The effect of PN on cell cytotoxicity was determined using the LDH assay method as described by Ref. [15]. In brief, cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and incubated overnight. They were then treated in triplicate with PN concentrations of 10, 50 and 100 μM and incubated for another 24 h. The LDH released from damaged cells was measured by Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The background control values were subtracted from each well and the mean percent treatment induced cytotoxicity for each cell line was calculated using the following equation:

$$100 \times [(\text{experimental value} - \text{low control})/ \times (\text{high control} - \text{low control})],$$

where: low control = mean absorbance from the untreated cells (spontaneous release of LDH) and high control = mean absorbance from lysis cells (maximum release of LDH) (positive control).

2.7.3. Measurement of DNA fragmentation

Cell death detection ELISA plus kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to quantify apoptosis-specific DNA fragmentation as described by Ref. [21]. After incubation with PN, cells were lysed for 30 min at room temperature and followed by centrifugation at 200*g* for 10 min. Then, 20 μl supernatant were transferred onto the streptavidin-coated plate, and 80 μl freshly prepared immune-reagent was added to each well and incubated for 2 h at room temperature. After the samples were washed with incubation buffer, the substrate solution was added and incubated for 20–30 min. The absorbance at 405 nm (reference wavelength 490 nm) was measured using a microplate reader. DNA fragmentation was expressed as the enrichment factor using the following equation:

$$\text{Enrichment factor} = (\text{Absorbance of the sample})/ \times (\text{Absorbance of the control})$$

where the sample is cells treated with PN and the control is cells without PN but vehicle alone (culture medium). Three independent experiments were carried out.

2.8. Western blot analysis

The PC-3 and LNCaP cells were seeded in T25 flasks, treated with PN at concentrations of 50 and 100 μM for 12 h, and then washed with PBS. Cell lysates were prepared using fresh standard lysis buffer for Western blot containing mixture of soybean trypsin inhibitor (100 μg/ml), aprotinin (10 μg/ml), phenylmethanesulfonyl fluoride (PMSF) (1 mM), EDTA (1 mM), SDS (1%), Tris-HCl (50 mM) and double-distilled water. Protein content was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL USA). Samples with equal amounts of protein (30 μg) from the lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (90 mA, 1 h 30 min). The membranes were blocked with 2% non-fat milk in TBS/0.1 Tween-20 at room temperature for 1 h. The TBS buffer contains: 20 mM Tris–HCl, 137 mM NaCl, and 0.1% Tween 20, pH 8.0. The membranes were incubated with specific primary antibodies against caspase-3 (a mixture of polyclonal rabbit anti-cleaved-caspase-3 (#9661) and polyclonal rabbit anti-caspase-3 (#9662) from Cell Signaling Technology, Danvers, MA, USA) and caspase-8 (a monoclonal mouse anti-Caspase-8 (#9746) from Cell Signaling Technology, Danvers, MA, USA) and were incubated overnight at 4 °C. After washing steps with TBS/0.1% Tween-20 twice (short) and thrice (long), and incubation with peroxidase-conjugated appropriate secondary antibodies (a polyclonal goat anti rabbit immunoglobulins/HRP (#P0448) for caspase-3, and a polyclonal rabbit anti mouse immunoglobulins/HRP (#P0260) for caspase-8 from DAKO Inc., Glostrup, Denmark). The blots were developed using the enhanced chemiluminescence (ECL) kit (Amersham Life Science, Pittsburgh, USA). The intensity of bands were determined using Fusion-Capt Advance Software (Vilbert Lourmat, Marne-la-Valleé, France).

2.9. Chick chorioallantoic membrane assay for angiogenesis

Fertilized chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) were kept in an incubator at 37 °C in constant humidity for 3 days. After day 3, a square window was cut into the shell of each egg to confirm a living embryo. The window was sealed with tape, and the eggs were kept in the incubator for additional 5 days. On day 8, the tapes were removed and the CAMs were treated with PN (10 and 50 μM) as described by Ref. [29]. In brief, two small silicone rings were placed onto one CAM and either 30 μl of vehicle (negative control) or 30 μl of PN (10 or 50 μM) were added topically. *In vivo* pictures were taken after 24 and 48 h using a stereomicroscope (Wild-Heerbrugg, Gais, Switzerland) equipped with a Kappa digital camera system (Kappa optronics GmbH, Gleichen, Germany). The response to drug treatment was assessed by examining the alteration in CAM of test groups and controls, and also by counting the numbers of blood vessels within the ring on each CAM.

2.10. Statistics

Statistical analysis was performed using the Microsoft Office 2003 Excel software and SPSS statistical software (Version 10.0). Statistical significance at $p < 0.05$ among treated, control and untreated groups were analysed by one-way ANOVA and Duncan post-hoc test. Values were expressed as mean ± standard deviation.

3. Results

3.1. Effects of PN on cell viability in prostate cancer cells

PN inhibited the proliferation of prostate cancer (PCa) cells in a concentration- and time-dependent manner (Figs. 1 and 2). At 10, 50 and 100 μM of PN, LNCaP was inhibited by 30%, 60% and 70% (Fig. 1), while PC-3 at the same concentration was inhibited by 28%, 52% and 55%, respectively after incubation for 96 h (Fig. 2). The cytotoxicity of PN was measured by quantification of LDH activity released from damaged cells after treatment. Cells were incubated with 10, 50 and 100 μM of PN for 24 h. Data analysis of results showed that there were no significant differences ($p > 0.05$) in LDH activities in BPH-1, PC-3 and LNCaP-treated cells relative to control after incubation with PN for 24 h at all concentrations (Table 1). The result indicates that the membrane of PCa cells remained intact during incubation with PN at the chosen concentrations. This was further confirmed by the insignificant effect ($p > 0.05$) of PN at different concentrations on the viability of BPH-1 cells (Fig. 3).

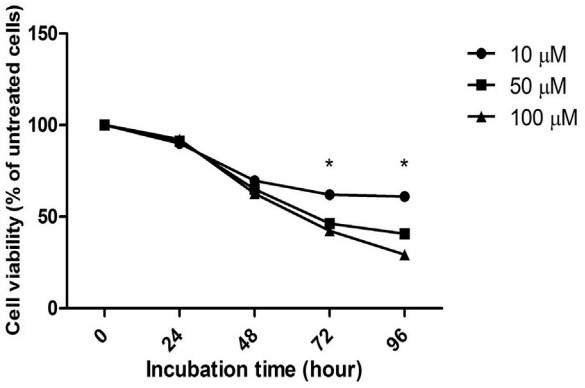

Fig. 1. Effects of punicalagin on the proliferation of LNCaP cells. *Significantly different from control ($p < 0.05$).

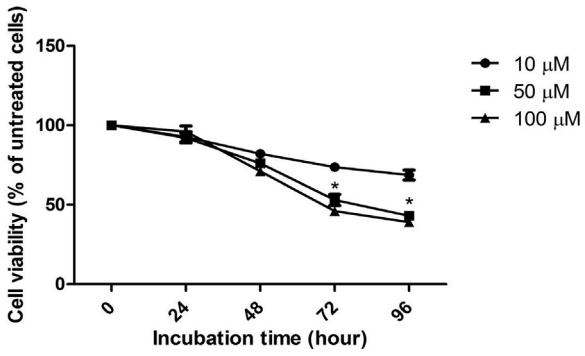

Fig. 2. Effects of punicalagin on the proliferation of PC-3 cells. *Significantly different from control ($p < 0.05$).

Table 1
Cytotoxicity of Punicalagin (PN) on PC-3, LNCaP and BPH-1 cells measured with LDH toxicity assay kit.

Treatment (μM)	Cytotoxicity (%) after 24 h incubation		
	PC-3	LNCaP	BPH-1
Control	0.86±0.07	0.82±0.14	0.89±0.12
PN 10	1.04±0.05	0.97±0.16	0.80±0.17
PN 50	1.12±0.05	1.11±0.23	1.08±0.05
PN 100	1.01±0.08	1.01±0.15	1.02±0.22

Data are expressed as Mean ± S.D. for 3 different experiments.
PN 10 = Punicalagin at 10 μM, PN 50 = Punicalagin at 50 μM.
PN 100 = Punicalagin at 100 μM.

3.2. The antioxidant effect of PN in vitro

PN concentration-dependently (10–200 μM) scavenged DPPH radical. At 10, 100 and 200 μM, PN scavenged DPPH radical by 7, 52 and 75%, respectively. The scavenging effect of PN was statistically similar ($p > 0.05$) to the standard (catechin) at 200 μM (Table 2). The percentage inhibition of lipid peroxidation by PN increased with increase in concentration. At 10, 100 and 200 μM of PN, the inhibition of lipid peroxidatin by PN were 15, 61 and 74%, respectively (Table 2).

3.3. PN induces cell death in LNCaP and PC-3 cells

The effects of different concentrations of PN on cell death were determined in PC-3 and LNCaP using Cell death Detection ELISA and Western blot assays. Cells were treated with different concentrations of PN for 24 h. Results indicated that cell death in PC-3 increased at higher concentrations of PN (50 and 100 μM), while significant increase in cell death was found in LNCaP cells treated with 100 μM of PN (Table 3). Western blot analysis revealed that PN at 50 and 100 μM induced the expression of caspase-3 (Fig. 4a) and caspase-8 (Fig. 4b) in PC-3 after 12 h incubation. However, PN produced insignificant effects ($p > 0.05$) on the expression of caspases-3 and -8 in LNCaP (Fig. 4).

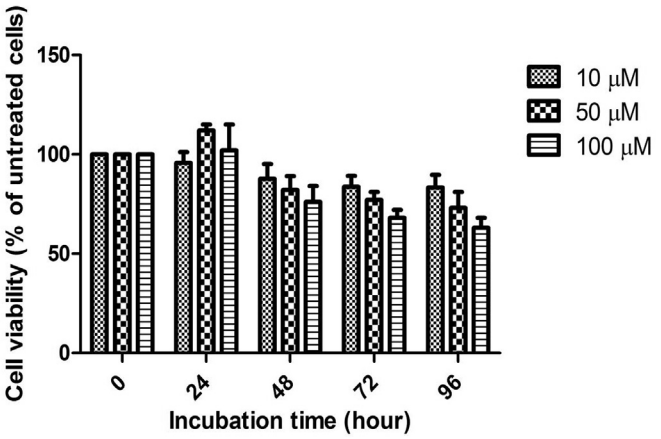

Fig. 3. Effects of punicalagin on the proliferation of BPH-1 cells.

Table 2
Scavenging activity of Punicalagin (PN) on 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical and inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation (LPO *in vitro*).

Grouping PN (μM)	Scavenging of DPPH (%)		Inhibition of LPO (%)	
	Catechin	PN	Catechin	PN
Control	0.00	0.00	0.00	0.00
10	5.3±0.04*	7.3±0.05*	23.4±1.05*	15.3±0.73*
50	19.3±0.32*	25.2±0.60*	44.5±2.35*	35.3±1.22*
100	38.2±0.75*	52.1±2.14*	64.3±2.02*	60.8±2.31*
150	59.1±2.11*	67.6±1.82*	76.1±3.43*	72.8±2.57*
200	68.4±2.50*	74.9±2.37*	78.2±3.21*	73.7±1.76*

Data are expressed as mean ± SD for 3 different experiments.
* Dose-dependent and significant increase from 10 to 200 μM of PN ($p < 0.05$).

Table 3
Effects of different concentrations of Punicalagin (PN) on apoptosis in LNCaP and PC-3 cells after 24 h exposure measured with Cell Death Detection ELISA kit.

Treatment (μM)	Enrichment factor	
	LNCaP	PC-3
Control	1.00±0.00	1.00±0.00
PN 10	1.15±0.04	1.18±0.05
PN 50	1.27±0.08	1.91±0.01*
PN 100	2.31±0.26*	2.34±0.05*
Positive Control	24.08±0.89*	21.11±0.16*

Data are expressed as mean ± SD for 3 different experiments.
* Significantly different from control ($p < 0.05$) PN.
PN 10 = Punicalagin at 10 μM, PN 50 = Punicalagin at 50 μM.
PN 100 = Punicalagin at 100 μM.

3.4. In vivo evaluation of angiogenic effects of PN

Fig. 5 depicts the anti-angiogenic effects of PN using the chicken chorioallantoic membrane (CAM) assay. Control CAMs have regular networks of vessels that consisted of a continuously perfused arteries and veins with adjacent capillary bed. Topical administration of PN (50 μM) to the CAM induced changes in the structure of the

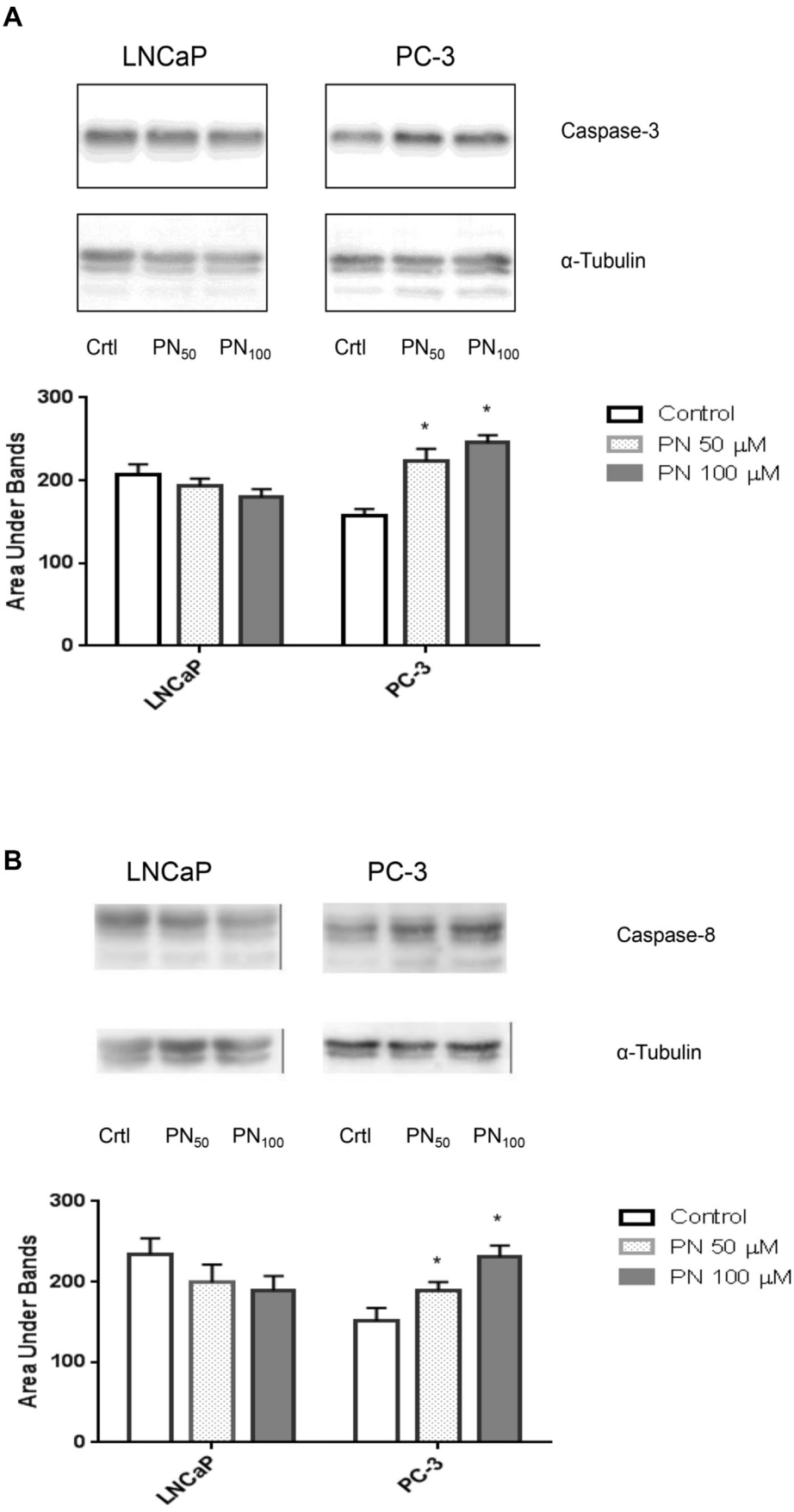

Fig. 4. A. Immunoblotting of caspase-3 levels in LNCaP and PC-3 cells untreated and treated for 12 h with punicalagin (PN) at 50 and 100 μM. Values are expressed as densitometric units corresponding to area under bands. *Significantly different from control ($p < 0.05$). **B**. Immunoblotting of caspase-8 levels in LNCaP and PC-3 cells untreated and treated for 12 h with punicalagin (PN) at 50 and 100 μM. Values are expressed as densitometric units corresponding to area under bands. *Significantly different from control ($p < 0.05$).

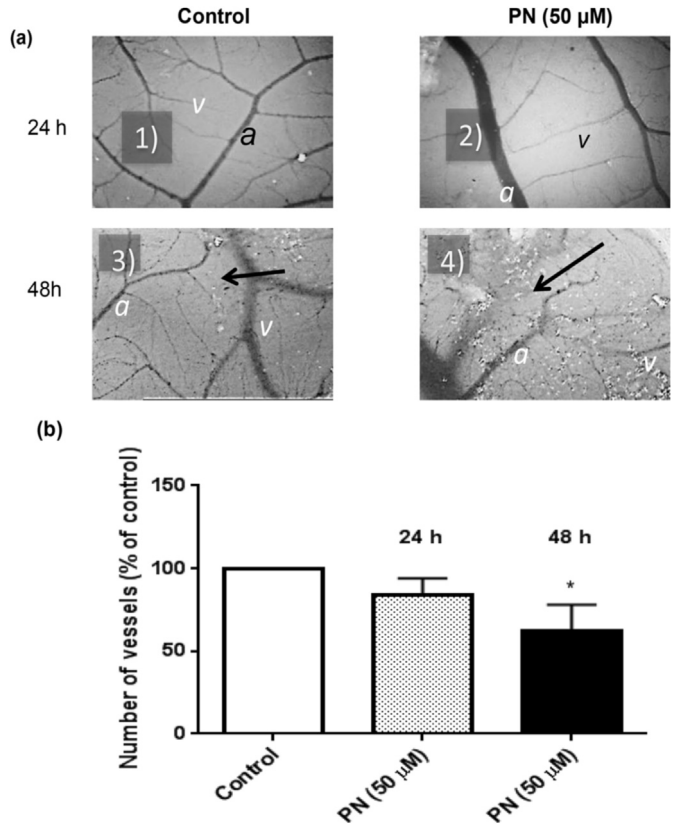

Fig. 5. PN induced changes in vascular bed of CAM. Two areas of the CAM were separated by placing two small silicone onto one CAM on day 8 of chick embryo development and control vehicle or 50 μM PN were applied topically. **a**: Representative microphotographs (x12) of control area (1, 3) and PN-treated area of the same CAM were shown after incubation for 24 h (2), and incubation for 48 h. Symbols: a = artery; v = vein. Vessel structure and diameter is regular in the control CAMs (arrow) (1, 3). 50 μM of compound PN changes the diameter of the existing vessels and reduces new vessel development seen as a vascular area of the CAM (arrows). Representative images from a total of 4 CAMs are shown. **b**: Quantification of blood vessels. Vessels were counted in six sections of each CAM and changes are shown as percentage in relation to control CAM (mean ± SD).

vascular network. After 24 h, the blood vessel growth was inhibited and, the perfusion of PN-treated area was uneven and decreased. Moreover, after 48 h of incubation, the regular structure of the vascular network was disrupted and, the numbers of perfused vessels were markedly reduced when compared to untreated CAM (Control) (Fig. 5).

4. Discussion

The quest for effective and safe drug candidates for treatment of cancers is urgently necessary in view of the fact that many anticancer drugs elicit harmful side effects on normal cells [39]. Ideally, anticancer therapies should have selective cell-killing effects. In the present study, we found that PN showed selective growth inhibition towards PC-3 and LNCaP cells, but was less harmful to the normal BPH-1 cells. Therefore, PN has the potential for selective killing towards PCa cells. In addition, PN inhibited the growth of PC-3 at concentrations of 50 and 100 μM, while LNCaP was inhibited in a concentration-dependent manner (10, 50 and 100 μM), hence as the concentration of PN increased, the number of live-cells decreased. At 100 μM PN, the cell viability was 27% and 35%, respectively for LNCaP and PC-3 after 96 h incubation, thus the polyphenol was effective against PCa cells. Furthermore, the high percentage of live-cells observed in BPH-1, low percentage of live-cells in PCa cells as well as low activity of LDH in the culture medium after incubation with PN confirmed that the polyphenol elicited antiproliferative (and not cytotoxic) activity against PCa cells. The results suggest that PN-induced growth inhibition was not mediated by the destruction of membranes of PCa cells but by intrinsic biochemical changes.

Antioxidants have attracted much interest for their protective effect against free radical-induced damage associated with many diseases including cancer. Assays such as DPPH radical scavenging, is among the most popular spectrophotometric methods for determination of antioxidant capacity of compounds, foods, beverages and vegetable extracts [16]. In this study, PN exhibited potent antioxidant activity as revealed by its high scavenging effect on DPPH radical and inhibition of lipid peroxidation (LPO) in rats' liver homogenate. PN showed concentration-dependent scavenging of DPPH radical and inhibitory effect on LPO reactions. This observation is consistent with the study of [25] and [7]. It is well-known that phenolic groups stabilize radicals formed on phenolic carbons with their resonance structure. PN has many phenolic rings and, an abstraction of hydrogen atom from phenolic hydroxyl group may occur easily [17]. Thus, PN may terminate free radicals reaction in a biological system.

Apoptosis, a suicidal cell death mechanism, is a well controlled process that gets activated either by the intrinsic or the extrinsic pathways [12]. The cells undergoing apoptosis exhibit caspase mediated cell death through transforming pro-caspases into active caspases [12]. Whereas the extrinsic pathway gets activated with the binding of Fas ligands to Fas proteins, the intrinsic pathway is triggered by redox changes within cells releasing the cytochrome-c from the disrupted mitochondria [32]. Thus, apoptosis is used by the body to regulate the proliferation of cells [36]. Therefore, an imbalance in pathways that regulate apoptosis can result in the formation of tumours and cancer progression [36]. Universally, DNA cleavage is an acceptable sign of apoptosis and, DNA fragmentation assay was conducted on the treated PCa cells in this study to confirm apoptosis. The apoptotic effect confirmed by the DNA fragmentation showed that PN treatment increased DNA fragments formation in PCa cells, suggesting that the cells treated with PN underwent apoptosis. These results show that PN induced DNA fragmentation in both hormone -dependent and -independent prostate cancer cells. Therefore, PN may be useful as a chemotherapeutic agent in the management of advanced prostate cancer. Further, we confirmed whether the apoptosis induced by PN was mediated through caspase activation or not, using Western blotting. Caspase-3 is known as effector caspase, and after being activated by the initiator caspases (caspase-8), it induces apoptosis. It was observed that the treatment of PC-3 cells with PN at 50 and 100 μM caused a significant increase in expression of both caspases-3 and -8, while the expression of these caspases was not affected in LNCaP cells. From these results, it was confirmed that PN induced apoptosis through caspase-3 activation in PC-3. Thus, caspases could play a central role in the activation of the executioner phase of PN-induced apoptosis.

Angiogenesis is a biochemical event associated with tumor cell migration and invasions. These are strategies developed and adopted by cancer cells to localize into distant organs and to escape drug treatment [11]. Metastatic tumor cells are hard to treat due to their ability to circulate in the blood as well as unusual expression of oncogenic proteins, and as such better strategies are required to prevent the metastatic spread [18]. One such strategy is to use agents which are known to halt the migration of cells by interfering with epithelial-to-mesenchymal transition, vascularization of tissues, cell invasion and extra-vasation [41]. In this study, PN at a concentration of 50 μM led to the inhibition of vascular network in

CAM; thus demonstrating a direct effect of PN on angiogenesis. This result is in line with the study of [9] which reported that norcantharidin, a synthetic antitumor compound, exhibited anti-metastatic and anti-angiogenic activities via the inhibition of migration and capillary-like tube formation of human umbilical endothelial cells.

In conclusion, this study demonstrates pronounced anti-proliferative and apoptosis-inducing effects of PN in prostate cancer cells. The effects included the scavenging of DPPH radical, and a generally reduced cellular viability of PCa cells, while non-cancerous prostate cells were less affected. Thus, PN may become an interesting compound for the treatment of PCa. Additional studies are required to further characterize the exact modes of action of PN in PCa cells before *in vivo* testing with an animal experimental model.

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Transparency document

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Declarations of conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

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