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ORIGINAL PAPER

Pomegranate Juice and Peel Extracts are Able to Inhibit Proliferation, Migration and Colony Formation of Prostate Cancer Cell Lines and Modulate the Akt/mTOR/S6K Signaling Pathway

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Abstract

Pomegranate (*Punica granatum*) is known to contain polyphenols with many potential health benefits, including anti-tumoral, anti-inflammatory, and anti-microbial properties. It has been used in popular medicine for cancer treatment, which still represents the major cause of cancer-related deaths in men worldwide. Importantly, pomegranate peels are valuable by-products of the food industry that are rich in polyphenols. Here we report a comparison between juice and peel aqueous extracts in prostate cancer DU-145 and PC-3 cell lines. Both extracts were able to inhibit the proliferation, migration and colony formation of those cells, although peel extracts presented more robust effects compared to juice. Besides, the growth-related mTOR/S6K signaling pathway presented strong inhibition after pomegranate extracts treatment. This study presents evidence that both juice and isolated peel extracts from promegate fruit have important anti-cancer effects against prostate cancer cells, modulating the mTOR/S6K signaling pathway.

Keywords Pomegranate · Peel extracts · Ellagic acid · Prostate cancer · mTOR · Cell signaling

Abbreviations

mTOR	Mammalian Target Of Rapamycin
FBS	Fetal bovine serum
PLC	High-Performance Liquid Chromatography
TRAMP	Transgenic adenocarcinoma of the mouse prostate

Introduction

Despite recent advances in research, prostate cancer still represents a major cause of cancer-related morbidity and mortality in men worldwide [1]. The mTOR (mammalian target of rapamycin) signaling pathway is an important controller of cell energy expenditure, signaling to protein synthesis and cell growth and considered an important modulated pathway in cancer [2, 3]. Some natural compounds, when evaluated in early stages of clinical trials, have been shown to be tolerated, safe and effective against several cancer types, generating several efforts in using these compounds for cancer therapy [4]. Moreover, some natural products can reduce adverse effects caused by chemotherapy and radiotherapy, responsible to reduce the quality of life in cancer patients [5].

Pomegranate (*Punica granatum*) is a member of the Lythraceae family and contains polyphenols with many potential health benefits, such as antioxidant, anti-microbial, anti-inflammatory and anti-cancer properties, including action in the cell cycle, proliferation, invasion and angiogenesis [6]. Pomegranate fruit, including juice, peels, and oil, has been traditionally used in popular medicine against cancer in

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different cultures and societies for centuries [7, 8]. According to Turrini et al. [9], pomegranate presents relevant beneficial effects, among several other natural compounds used for the prevention and treatment of prostate cancer.

Ellagitannins (ex. punicalagins), punicic acid, flavanoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones are therapeutically known significant constituents of pomegranate [10]. Ellagitannins belong to the polyphenol group of hydrolyzable tannins [10]. After their hydrolysis, they liberate ellagic acid and after microbiota assisted processing they also liberate urolithins [11]. Ellagitannins, including ellagic acid, are bioactive polyphenols present in the pomegranate fruit juice that have chemopreventive potential against multiple human carcinomas [12]. The cytotoxic activity has also been related to ursolic acid, ellagic acid, luteolin, and punicic acid from Punica extracts [10].

Several pre-clinical and clinical research studies present evidence of the anti-cancer effects of pomegranate extracts, most using juice-based extracts against cancer cell lines [10, 12]. This report aimed for a comparison between juice and peel aqueous extracts in prostate cancer DU-145 and PC-3 cell lines, assessing proliferation, migration, colony formation of those cells and the Akt/mTOR/S6K signaling pathway status.

Material and Methods

Cell Culture

PC-3 human metastatic prostate cancer cells and DU-145 human metastatic prostate cancer cells were grown as previously described [3].

Pomegranate Extracts Preparation

1 g of lyophilized peels extract or lyophilized pomegranate juice was placed in 10 mL of deionized water. The mixture was homogenized for 1 min on a vortex. The extraction was performed in an ultrasonic bath at 39 °C for 45 min. The extracts were then centrifuged (TDL80-2B) at 5000 rpm for 10 min at 4 °C and subsequently filtered to obtain aqueous extracts. The quantification of total phenolic content was based on a method published by Singleton et al. [13]. The absorbance was measured at 740 nm using a microplate spectrophotometer (Biotek Instruments, Germany). The total phenol content was calculated using a curve prepared with gallic acid (calibration range 20–120 μg/mL) and expressed in terms of gallic acid equivalents *per* dry weight (mg of GAE/g of DW).

Antioxidant Activity of Pomegranate Peel and Juice Extracts

The antioxidant activity of pomegranate extracts was analyzed by the ferric reducing antioxidant power (FRAP) method, based on the Benzie and Strain methodology [14]. The antioxidant activity was expressed as ferrous sulfate equivalents *per* dry weight (μmol of Fe^{+2}/g of DW) and calculated using the calibration curve (calibration range 100–700 μM).

Quantification of Phenolics Content by Chromatography

The extracts were analyzed by high-performance liquid chromatography in the EXTRAC-US system (FAPESP 2013/04304–4 – patent pending). Separation of the compound found in the sample was performed with a C_{18} column (Kinetex C18, 2.6 μm, 100 A, 100 × 4.6 mm, Phenomenex, Torrance, CA, USA). Chromatograms were recorded at 370 nm. These conditions were based on previously developed methods [15]. The identification of the compounds present in the extracts was based on the comparison of the retention time of authentic standards of punicalagin and ellagic acid (Sigma Aldrich, USA). Results were expressed as mg of phenolic compound/L of extract and the concentration of unidentified peaks was as equivalents of other phenolic compound/L of extract.

Proliferation Test

Cultures of PC-3 and DU-145 cells were plated in 24-well plates at 5×10^4 cells / well and incubated with extracts for 24 h or 48 h with 10% FBS at 37 °C. Counts were performed with an automated cell counter (Invitrogen) and Trypan blue exclusion staining.

Scratch Migration Assay

Cultures of PC-3 and DU-145 cells were seeded at a density of 5×10^5 cells/well in 6-well plates and incubated until confluence. Thereafter, the incubated cell cultures were scratched with a p200 pipette tip in the middle of the wells and the culture media were replaced by serum-free media [3]. The scratch area was analyzed by microscope and images were captured at times 0 h, 24 h, and 48 h. The scratch areas were quantified using ImageJ software.

Colonies Formation Assay

Cultures of PC-3 and DU-145 cells were plated at low density (5×10^3 cells/dish) in 60 mm dishes and treated for 10 days, with changes in media containing pomegranate extracts every two days. The cells were washed with PBS, stained with

methylene blue (3%) in methanol for 30 min and counted. Colonies smaller than 1 mm in diameter were excluded from counting [3].

Western Blotting

Proteins, in a total of 30 μg *per* sample, were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto nitrocellulose membranes. Western blotting was performed as previously described [3]. The following antibodies and dilutions were used: anti-pS6K1-Thr389; anti-S6K1; anti-pS6-Ser240/244; anti-S6; anti-pAkt-Ser473; anti-Akt; anti-pmTOR-Ser2448; anti-mTOR (Cell Signaling Technologies), diluted 1:1000; and anti-α-tubulin (Calbiochem), diluted 1:5000.

Statistical Analysis

The values presented are means ± standard deviation (SD). The statistical analyses were evaluated by ANOVA tests followed by Bonferroni's or Dunnet's *post*-test, using the software GraphPad Prism 5. The values of $p < 0.05$ were considered significant. Outliers test.

Results and Discussion

The total phenolic content and the antioxidant activity of the aqueous extracts of the juice and pomegranate peel were quantified by the Folin-Ciocalteau reagent for the polyphenols and by the iron ion reduction power for the antioxidant capacity and results were 390.77 ± 4.64 and 2043.33 ± 101.52 μmol of

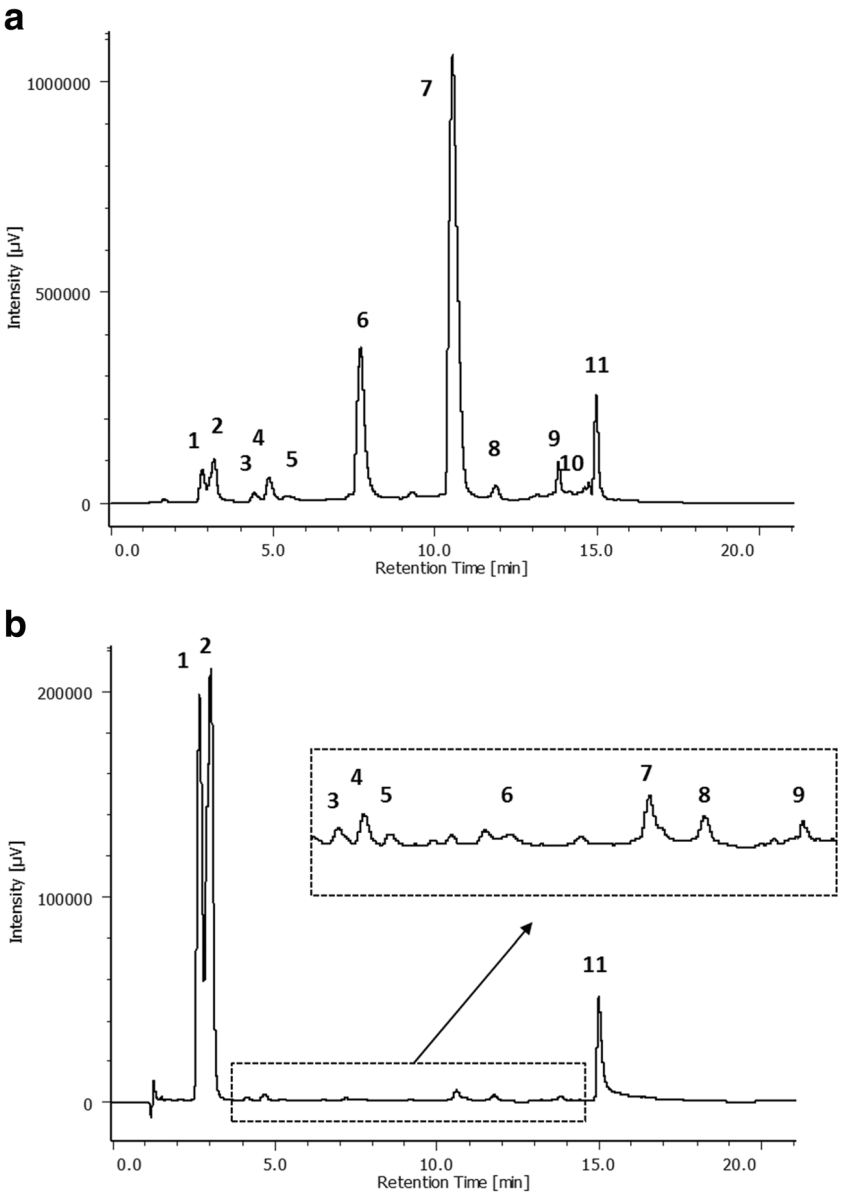

Fig. 1 Representative chromatogram recorded at 370 nm of the peel extract (**a**) and pomegranate juice (**b**) used in the study. Peaks #1–5: Unidentified compounds; Peak #6: α-Punicalagin; Peak #7: β-Punicalagin; Peak #8: Ellagic acid hexoside; Peak #9: Ellagic acid pentoside; Peak #10: Ellagic acid deoxyhexoside; Peak #11: Ellagic acid

Table I Compounds present in the pomegranate juice and peel extracts used in this study

Peak #	RT	Compound	Concentration (mg/L)	
			Juice	Peel Extract
1	2.81	Unidentified	593.9*	230.3*
2	3.17	Unidentified	794.8*	372.1*
3	4.41	Unidentified	6.0*	61.8*
4	4.87	Unidentified	11.2*	212.4*
5	5.41	Unidentified	3.8*	68.2*
6	7.68	α-Punicalagin	7.2	1647.9
7	10.51	β-Punicalagin	18.8	5715.1
8	11.84	Ellagic acid hexoside	0.6**	23.0**
9	13.77	Ellagic acid pentoside	tr	35.2**
10	14.71	Ellagic acid deoxyhexoside	n.d.	11.7**
11	14.93	Ellagic acid	51.8	138.6
		TOTAL	1488.2	8516.2

*Concentration expressed as mg of α-punicalagin equivalents/L

** Concentration expressed as mg of ellagic acid equivalents/L

RT Retention time; *Tr* Trace concentration; *n.d.* Not detected

Fe^{+2}/g of DW for juice and peel extract, respectively, and 23.87 ± 0.34 and 136.27 ± 1.34 mg of AGE/g of DW, for juice and peel extract, respectively. Thus, peel extracts presented higher concentrations of phenolics and FRAP values compared to juice. The extracts were further characterized for the main phenolics composition by liquid chromatography. Chromatograms of the juice and peel extracts are shown in Fig. 1, respectively, and the concentration of compounds present in the extract is presented in Table I.

The main phenolic compounds identified in the samples were α and β-punicalagin, ellagic acid and three of its derivatives (hexoside, pentoside, and deoxihexoside). In the peel extract there was a predominance of punicalagin followed by high amounts of ellagic acid and its derivatives, the pomegranate juice had a higher relative concentration of two very polar unidentified compounds (peaks #1 and #2 - Fig. 1 and Table I). With the exception of these two compounds the concentration of all identified compounds was lower in the pomegranate juice when compared to the peel extract. Only small amounts of punicalagin have been detected in the juice and the concentration of ellagic acid was almost three times lower than in the peel extract. The total concentration of the main

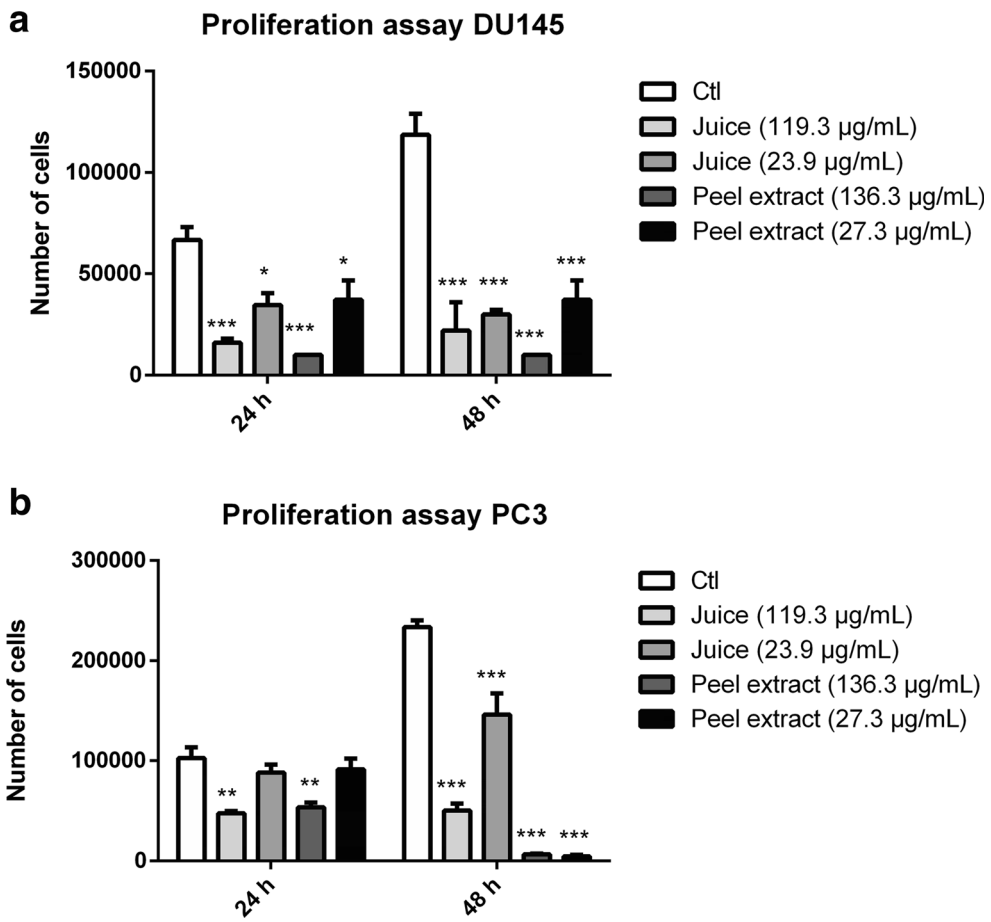

Fig. 2 Pomegranate juice and peel extract inhibit prostate cancer cell proliferation. Cells were seeded and treated with extracts for 24 and 48 h. Cells were counted using an automated cell counter. (**a**) Proliferation test of DU-145 cultures, (**b**) Proliferation test of PC-3 cultures. Data are presented as mean ± standard deviation (SD). 1 µg/mL = 1 µg of AGE/mL. Statistical analysis has been performed by two-way ANOVA and Bonferroni's *post*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$

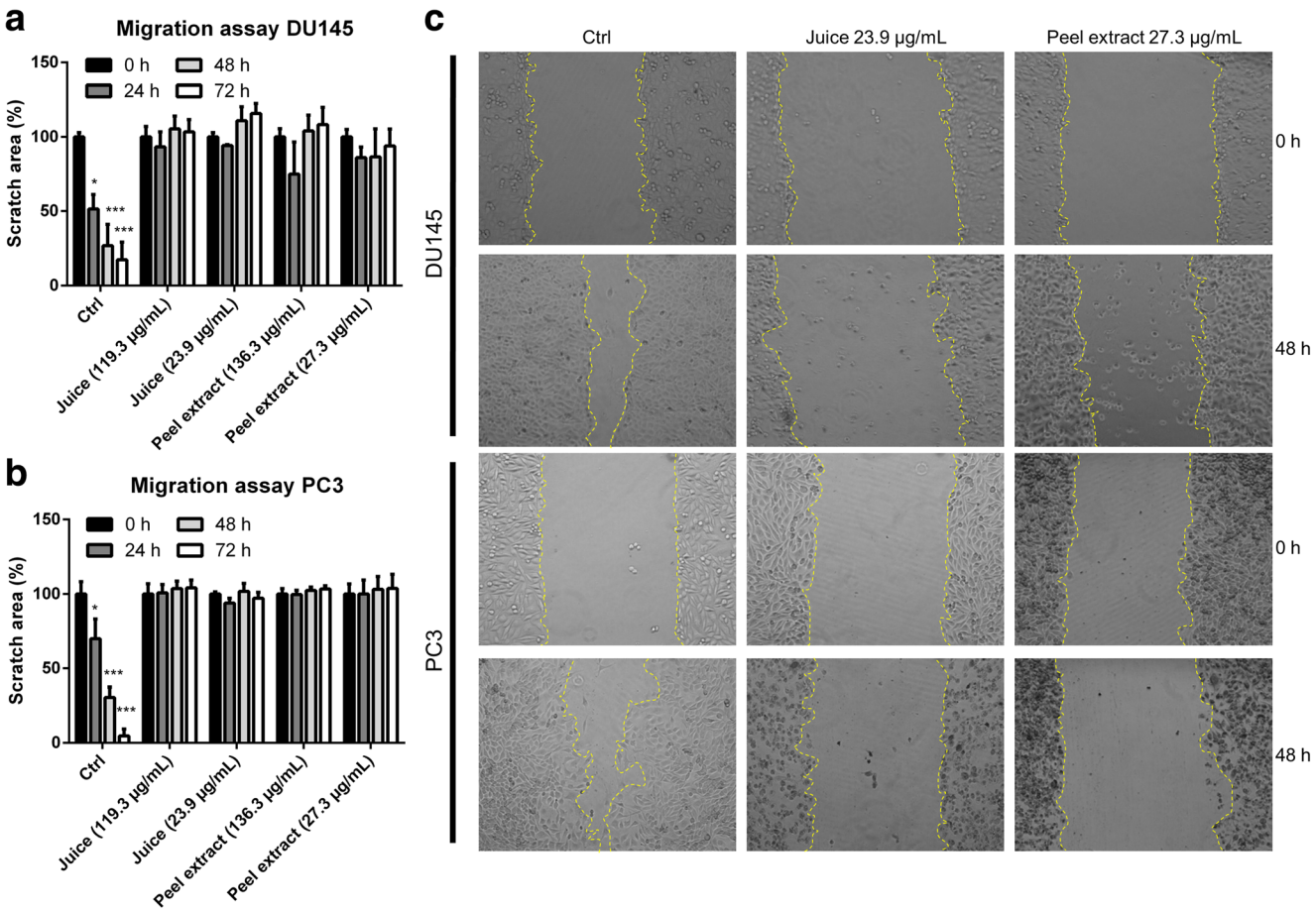

Fig. 3 Pomegranate juice and peel extract inhibit prostate cancer cell migration in a scratch assay. Cells were seeded and grown until confluence, scratched using a pipette tip and incubated with extracts in serum-free conditions. (**a**) Percentage of scratch areas of DU-145 cultures. (**b**) Percentage of the scratch area of PC-3 cultures, (**c**) Representative images of scratch areas of DU-145 and PC-3 cultures after at time 0 and 48 h. Data are presented as mean ± standard deviation (SD). 1 μg/mL = 1 μg of AGE/mL. Statistical analysis has been performed by two-way ANOVA and Bonferroni's *post*-test. $*p < 0.05$, $***p < 0.001$, $n = 3$

detected compounds in the peel extract was almost six times higher than in the pomegranate juice.

Interestingly, in terms of polyphenols, pomegranate peels are one of the most valuable by-products of the food industry [16]. A study has demonstrated that the enrichment of other fruit juices with dried extract of pomegranate peel increased antioxidant activity [17]. About 40–50% of the total pomegranate fruit weight corresponds to peel, which is a significant source of bioactive compounds such as flavonoids, phenolics, ellagitannins, punicalagin and punicalin [16]. Pomegranates peels and leaves are most traditionally used to treat stomach disorders and diarrhea, although several studies link its anti-inflammatory and antioxidant properties to cancer protection [18]. The chromatographic analysis revealed a phenolic profile of the peel extract and juice consistent with the information available in the literature, which reports higher concentration of punicalagin in the peels and mesocarps compared to the juice [19]. The high concentration of punicalagin in the peel extract is particularly relevant since it is a derivative of ellagic acid. Ellagic acid has demonstrated chemopreventive potential against prostate cancer, activating pro-apoptotic genes in an animal model [12]. Several other phenolic compounds, like resveratrol and epigallocatechin gallate, have been reported to have anti-tumorigenic effects against prostate cancer cells [20, 21].

In order to compare the effects of juice and peel extracts in prostate cancer cells, both were diluted at close concentrations and added to cultures of PC-3 and DU-145. After 24 and 48 h, all concentrations presented cytostatic effects in DU-145 cells (Fig. 2A), and only juice at 23.9 μg/mL and peel extract at 27.3 μg/mL did not inhibit PC-3 after 24 h (Fig. 2B). Peel extracts presented a more robust effect in PC-3 after 48 h than juice at similar concentrations.

To evaluate the effects of juice and peel extracts in the migration of prostate cancer cells, a scratch assay has been performed in serum-free conditions. All tested conditions show that juice and peel extracts are able to inhibit the migration of PC-3 and DU-145 cells in monolayer culture (Fig. 3A and B). Representative images of each culture at 0 and 48 h are presented (Fig. 3C), showing a prominent effect on cell migration. Migration is a feature

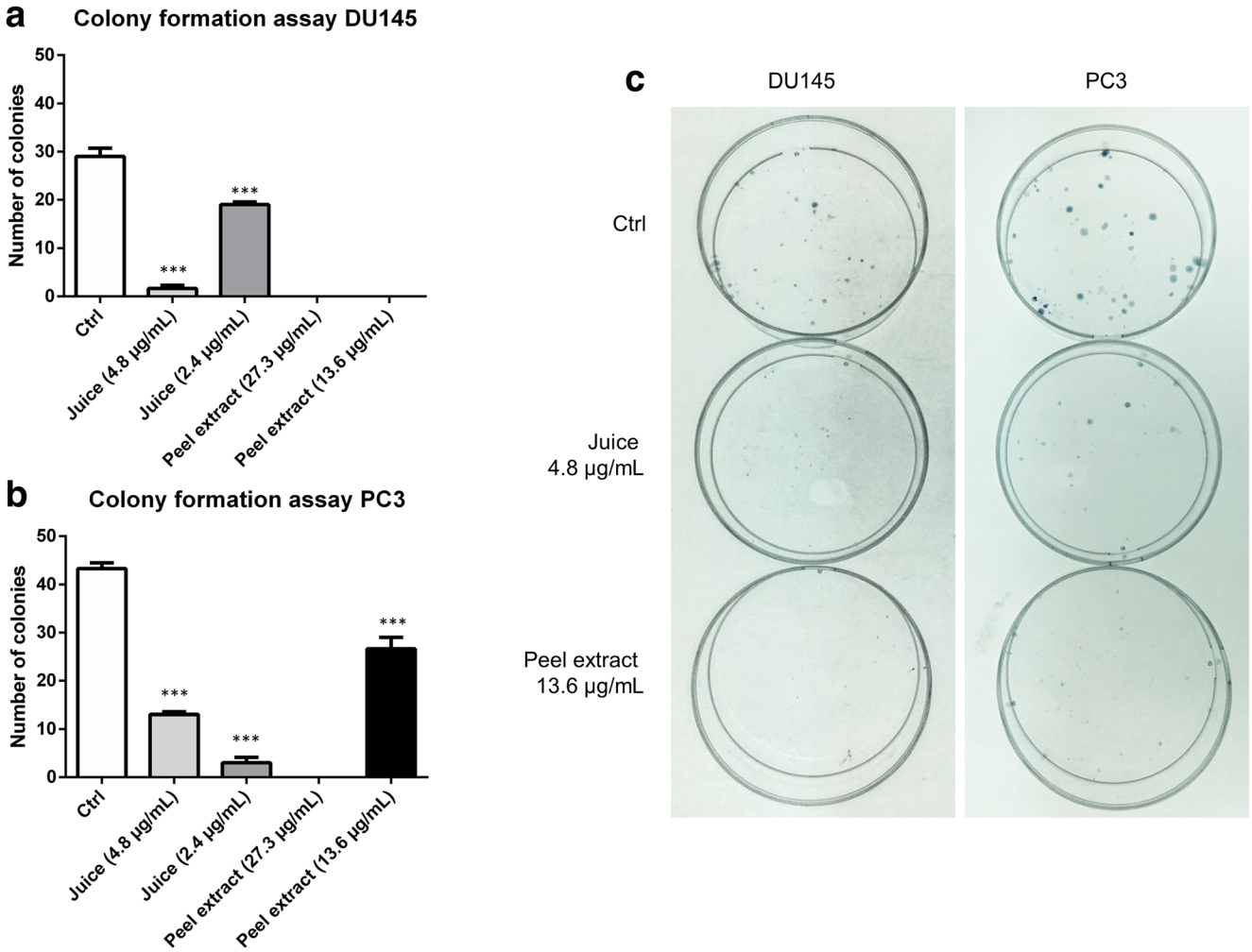

Fig. 4 Pomegranate juice and peel extract inhibit prostate cancer cell colonies formation. Cells were seeded at low density in P60 plates and grown for several days in the presence of extracts. (**a**) The number of colonies DU-145 cultures, (**b**) the number of colonies of PC-3 cultures, (**c**) representative images of DU-145 and PC-3 colonies stained with methylene blue. Data are presented as mean ± standard deviation (SD). 1 µg/mL = 1 µg of AGE/mL. Statistical analysis has been performed by one-way ANOVA and Dunnet's *post*-test. ***$p < 0.001$, $n = 3$

of cancer cells that is deregulated in advanced and metastatic stages of tumor progression and other studies have shown the potency of pomegranate extracts to inhibit migration of lung and ovarian cancer cells through impairment of metalloproteinases [22]. Albrecht et al. [23] studied pomegranate pericarp, fermented juice polyphenols and seed oil extracts on cell cycle, proliferation, apoptosis, gene expression regulation, invasion and *in vivo* prostate tumor growth [23]. DU-145 cell line presented a significant increase of 11% in G2/M cell cycle phase by treatment with seed oil. For PC-3 cells, all agents suppressed invasion through Matrigel. Pomegranate pericarp polyphenols and seed oil also demonstrated potent inhibition of PC-3 xenograft growth in athymic mice.

The effects of juice and peel extracts on colony formation have also been tested and results are presented in Fig. 4. Both PC-3 and DU-145 presented colony formation inhibition in the presence of pomegranate extracts, whereas peel extracts were more efficient for that inhibition (Fig. 4A and B). Representative images of each culture are presented in Fig. 4C. PC-3 cells present strong metastatic potential and are androgen-independent, and although DU-145 cells are also highly proliferative and androgen-independent, they have moderate metastatic capability [8]. Indeed our results show that PC-3 cells are more resistant to anti-cancer effects of pomegranate extracts compared to DU-145, as seen by proliferation assay and colony formation (Figs. 2 and 4).

In order to explore cell signaling mechanisms related to the effects of pomegranate treatments in prostate cancer cells, we have evaluated the mTOR signaling pathway status after 48 h of treatments. mTOR pathway was evaluated since it is a key controller of cell growth and metabolism and several studies report the deregulation of this pathway in prostate cancer [2, 3, 24]. Pomegranate juice has been diluted at 2.4 µg/mL and peels extract to 13.6 µg/mL. As seen in Fig. 5, significant reductions in phosphorylation levels of Akt, S6K1, and S6

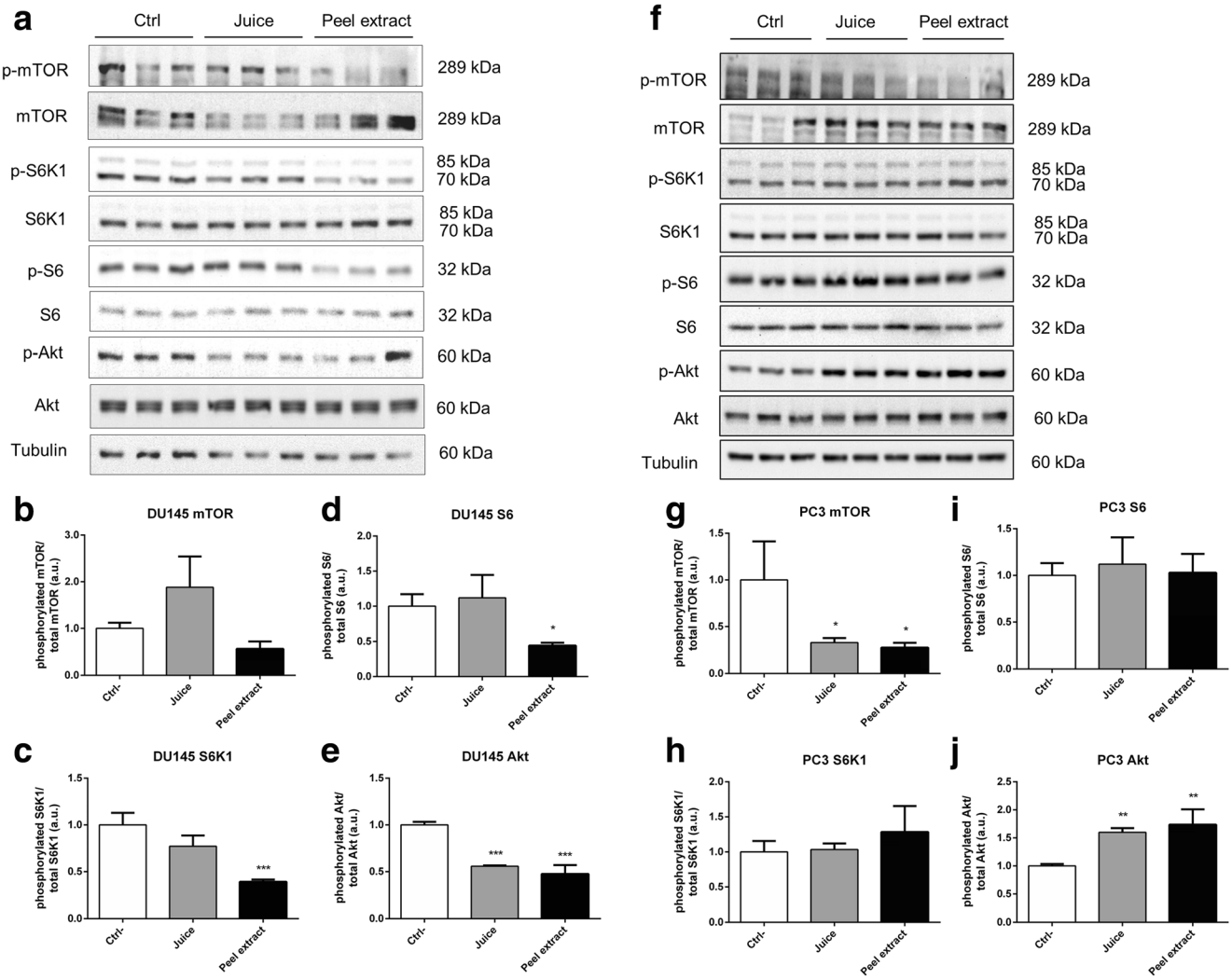

Fig. 5 Pomegranate juice and peel extract modulate the activation of the mTOR/S6K growth signaling pathway. (**a**, **f**) Western blotting of mTOR, Akt, S6K1, S6 and α-tubulin (control) of protein extracts from cells treated with Pomegranate juice diluted at 2.4 μg/mL and peel extract at 13.6 μg/mL after 48 h, (**b**, **g**) Normalized phosphorylation levels of mTOR, (**c**, **h**) Normalized phosphorylation levels of S6K1, (**d**, **i**) Normalized phosphorylation levels of S6, (**e**, **j**) Normalized phosphorylation levels of Akt, (**a**-**e**) Analyses for DU-145 cells, (**f**-**j**) Analyses for PC-3 cells. Data are presented as mean ± standard deviation (SD). Statistical analysis has been performed by one-way ANOVA and Dunnet's *post*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 3$

have been observed for DU-145 for the peel extract (Fig. 5A-E), including juice for Akt. For PC-3, inactivation of mTOR has been observed for both juice and peel extract (Fig. 5F-J).

The mTOR pathway can be inhibited directly or indirectly by several natural compounds, including phytochemicals present in pomegranate, and is frequently activated in several human cancers, being considered an attractive therapeutic target for cancer therapy [2, 4]. The consumption of pomegranate juice *ad libitum* for 10 weeks in rats suppressed the number of crypt foci and inhibited PI3K/Akt phosphorylation and mTOR expression in HT-29 colon cancer cell line [25]. In another study using rats, pomegranate extracts decreased p70S6K and RPS6, as well as Rps6ka2, Map 2 k2, and Mapk1 mRNA [26]. Ellagic acid presented in pomegranate peel extract is able to inhibit the Akt/mTOR signaling pathway by increasing the expression level of IGFBP7 [27]. Finally, inhibition of prostate cancer growth and metastasis due to oral supplementation with pomegranate fruit extract has been reported in transgenic TRAMP (transgenic adenocarcinoma of the mouse prostate) mice, most likely through inhibition of IGF-I/Akt/mTOR [28]. Punicalagin has been reported to inhibit the mTOR signaling pathway in other cell models, in agreement with the results presented in Fig. 5 [29].

As PC-3 has a robust activation of PI3K/Akt due to the PTEN loss, whereas DU-145 does not [30], inhibitory effects of pomegranate extracts in this pathway may be limited, as shown in Fig. 5. Several studies were also able to show different molecular responses between DU-145 and PC-3 [31, 32]. Besides, inhibition of the Akt/mTOR pathway may in part explain the reduction of proliferation, migration and colonies

formation in DU145 cells, since this is a central pathway in controlling apoptosis and autophagy [2]. These effects on the mTOR pathway, however, were not robust in PC-3 cells, even with changes in proliferation, migration and colony formation. Therefore, we were able to find molecular differences in the response to pomegranate extracts in those cell lines.

It is well known that diets rich in phytochemicals have been associated with a reduced risk of diseases including several types of cancer, inflammation, neurodegenerative and cardiovascular diseases [19]. Besides, there is an inverse association of cancer incidence/mortality and vegetable and fruit consumption [9]. Phase II clinical trials have associated consumption of pomegranate juice with prolongation of PSA (Prostate-Specific Antigen) doubling time in men diagnosed with prostate carcinoma [9]. Changes in microbiota have also been demonstrated after pomegranate consumption and microbiota-derived metabolites, like urolithins and ellagic acid, were demonstrated to reach the human prostate tissue [33].

Conclusions

In this study we show that juice and isolated peel extracts from pomegranate are able to inhibit proliferation, migration and colony formation of prostate cancer cell lines, regulating the mTOR signaling pathway, a master controller of cell growth and metabolism. Besides, we present strong evidence that aqueous extracts from pomegranate peels, usually a by-product in food processing, have stronger anti-cancer effects when compared to juice from pomegranate. These results may have an impact on the therapeutics of cancer and for food industry related to pomegranate.

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Author's Contributions FMC and LGS have performed cell culture experiments. ICBP has performed western blotting analysis. MAR has performed phenolics analysis. AECA, RMNB, and FMS have designed experiments and written the manuscript. The authors declare no conflict of interest.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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