REGULAR ARTICLE

EFFICACY OF PHYTONUTRIENTS FROM POMEGRANATE PEEL ON HUMAN OVARIAN CELLS *IN VITRO*

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ABSTRACT

Pomegranate fruit (*Punica granatum* L.) is rich in antioxidants with a content of bioactive substances with high medicinal value. Punicalagin, a polyphenol from pomegranate fruit, has been studied for its antioxidant, anti-proliferative and anti-cancer activities. Ovarian cancer is one of the most common cancers in the female reproductive organs and with high rate of lethality. While it is confirmed that pomegranate has significant beneficial effects on several types of cancer, there are few detailed reports on epithelial ovarian cancer. In accordance with the potential health-promoting effects of pomegranate, the aim of our study was to examine the *in vitro* effect of punicalagin and pomegranate peel extract at the different concentrations (12.5, 25, 50, 100, and 200 µg/mL) for 24 h on the human ovarian granulosa cell line HGL5 and human ovarian carcinoma cell line OVCAR-3. For this experiment, the ethanol extract from lyophilized pomegranate peel was prepared. The metabolic activity was determined by AlamarBlue™ cell viability assay, the secretion of steroid hormones was assayed by the ELISA method. The results showed a significant ($P \leq 0.001$) decrease in the viability of HGL5 cells after the addition of the highest concentration of punicalagin (200 µg/mL). The number of viable OVCAR-3 cells was not significantly ($P \geq 0.05$) affected compared to the control. On the other hand, the concentrations 25, 50, 100, and 200 µg/mL of pomegranate peel extract led to a significant decrease in the viability of OVCAR-3 cells but did not cause any significant ($P \geq 0.05$) changes in the viability of HGL5. Although our studies revealed an increase in the release of 17β-estradiol levels by HGL5 cells after punicalagin treatment at the concentration 50 ($P \leq 0.01$) and 100 ($P \leq 0.05$) µg/mL, progesterone secretion was not significantly ($P \geq 0.05$) affected. Also, the release of 17β-estradiol was significantly increased after the supplementation of pomegranate peel extract at the concentrations 50 ($P \leq 0.01$), 100, and 200 ($P \leq 0.001$) µg/mL. Furthermore, the levels of progesterone were significantly ($P \leq 0.05$) decreased at concentrations 12.5, 25, 50, and 100 µg/mL. In conclusion, pomegranate phytonutrients might be a promising modulator of secretion of steroid hormones and it might serve to be a potential chemoprotective agent, reducing viability of ovarian cancer cells.

Key words: punicalagin, pomegranate, ovarian cells, steroid hormones, cancer

INTRODUCTION

Dietary phytochemicals present in fruits and vegetables exhibit many beneficial properties, including chemotherapeutic agents owing to their antitumor activity (**Zhou *et al.*, 2016**). Recent studies have showed that phytonutrients such as polyphenols, flavonoids (e.g. isoquercitrin, rutin, quercetin) (**Michalcova *et al.*, 2019; Roychoudhury *et al.*, 2018**), ellagitannins (e.g. punicalagin, ellagic acid) (**Packova *et al.*, 2015, 2016**), and glycosides (e.g. amygdalin) (**Halenar *et al.*, 2017; Kolesar *et al.*, 2018; Kopcekova *et al.*, 2018; Kovacikova *et al.*, 2019; Kolesárová *et al.*, 2020**) may possess health-promoting effects as well as and the ability to ameliorate alterations of the reproductive system. Recently, several *in vitro* and *in vivo* experiments have revealed the beneficial physiological activities of pomegranate fruit and human studies have further indicated its promising potential as a protective agent against various diseases (**Kandys and Kokkinomagoulos, 2020**).
Pomegranate (*Punica granatum* L.) is a rich source of valuable nutritional substances, including flavonols, anthocyanins, phenolic acids, mainly gallic acid and ellagic acid, organic acids, condensed and hydrolysable tannins, especially ellagitannins such as punicalagin, punicalin, and gallotannins. These bioactive substances have been related with various beneficial properties against a number of diseases. Pomegranate peels contain a lot of phenolic compounds, minerals, and polysaccharides, while arils contain water (85 %), sugars, pectin, organic acids, phenolics, and anthocyanins. Proteins, crude fibers, vitamins, minerals, pectin, sugars, polyphenols, isoflavones, and the composition of the pomegranate seed oil is mainly linolenic and linoleic acids, lipids such as punicic acid, oleic acid, stearic acid, and palmitic acid **(Viuda-Martos *et al.*, 2010)**. More recently, clinical and preclinical studies have provided scientific evidence that pomegranates possess remarkable antioxidant (**Singh *et al.*, 2014**), anti-inflammatory **(Gonzalez-Trujano *et al.*, 2015)**, anti-cancer **(Syed *et al.*, 2013; Sharma *et al.*, 2017)**, anti-obesity **(Al-Muammar *et al.*, 2012)**, and neuroprotective **(Yuan *et al.*, 2016)** properties. The multiple health benefits of the pomegranates are considered mainly to be due to the presence of polyphenol punicalagin and other metabolites, such as flavonols and anthocyanins (**Cerda *et al.*, 2003**).
Punicalagin, a bioactive constituent belonging to the family of ellagitannins, is the most abundant polyphenol found in pomegranate peel with a molecular weight of 1084.71 g/mol (**Yao *et al.*, 2017**), and responsible for more than half pomegranate juice´s antioxidant properties (**Seeram *et al.*, 2005).** Following the digestion path, ellagitannins are converted by the intestinal flora into urolithins (**Viuda-Martos *et al.*, 2010)**. Punicalagin has been associated with beneficial impact to human health, including anti-oxidant (**Seeram *et al.*, 2005),** anti-inflammatory, cardio-protective (**El-Missiry *et al.*, 2015**), neuro-protective (**Yaidikar and Thakur, 2015**), pro-apoptotic and anti-cancer (**Zhang *et al.*, 2020**) activities. Punic acid, kaempferol, and β-sitosterol are phytoestrogens found in pomegranates, which are structurally similar to steroid hormone 17β-estradiol and have shown phytoestrogenic activity **(Choi *et al.*, 2006)**, thus reducing the hormonal effect of endogenous estrogens **(Papoutsi *et al.*, 2005)**. Steroid hormone 17β-estradiol, the most effective female estrogen, and its receptors (ERα and ERβ) play a critical role in the control of a plethora of biological responses that strongly affect several aspects of physiology, including risk factors for the initiation and progression of hormone-related cancers. Depending on the estrogen receptor subtypes, 17β-estradiol exhibits divergent effects on cancer cells (**Deroo and Korach, 2006**).
Ovarian cancer, one of the most common malignant tumors, is the leading cause of death from gynecological cancers in women (**Siegel *et al.*, 2018**). One of the strategies is to develop novel low-toxic anti-cancer agents. Therefore, dietary products enriched by bioactive

phytochemicals may be used as a potential nutritional strategy in slowing the progression of gynecological malignancy and may provide useful alternative therapeutic approaches (**Zhou *et al*., 2016**).
So far, little is known about the impact of pomegranate or punicalagin on the female reproductive system and its effect on ovarian steroidogenesis or tumorigenesis. Therefore, selected ovarian model cells were used to study anti-proliferative effects in ovarian cancer and the modulation of secretion of steroid hormones, with the aim to better understand the mechanism of action of the above-mentioned phytonutrients. The immortalized human granulosa cell line HGL5 and human ovarian epithelial carcinoma cell line OVCAR-3 have been previously described **(Michalcova *et al*., 2019; Baldovská *et al*., 2019)**.
The objective of the present study was to examine the cell viability and the secretion of selected steroid hormones after supplementation of a number of the concentrations of punicalagin and pomegranate peel extract ranging from 12.5 to 200 µg/mL using human ovarian cells HGL5 and OVCAR-3. Furthermore, we compared the efficacy of punicalagin and pomegranate peel extract.

MATERIAL AND METHODS

Ovarian cell lines

The human ovarian granulosa cell line HGL5 (ABM®, BC, Canada) was cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, MO, USA), supplemented with 10 % fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotics (Invitrogen, CA, USA), and incubated in a 5 % CO_2 incubator at 37 °C.
The human ovarian carcinoma cell line OVCAR-3 was obtained from the American Type Culture Collection (ATCC®, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco-BRL, MD, USA) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotics (Invitrogen, CA, USA), 1 % non-essential amino acids (Sigma Aldrich, UK), and incubated in a 5 % CO_2 incubator at 37 °C. The initial concentrations of cells before setting up the culture ranged from 10^4 to 10^5 cells per mL. The cells were grown in a standard T75 cell culture flask (Corning Life Sciences, NY, USA) to 80-90 % confluence.

Treatment for human ovarian cells

Punicalagin (Sigma-Aldrich, St. Louis, MO, USA) and pomegranate peel extract were used in this study. For this experiment, the ethanol extract from lyophilized pomegranate peel was prepared. Prior to the experiments, pure punicalagin was dissolved in a culture medium and diluted to the desired concentrations. Depending on the treatment, the cells were cultured in plates without (control group) or with punicalagin or pomegranate peel extract at concentrations 12.5, 25, 50, 100, and 200 µg/mL for short-term application (24 h). Cells treated with ethanol in an amount corresponding to the highest used concentration of the extract were used as positive controls (+Control) for the experiments.

Cell viability assay

Cell viability was evaluated using AlamarBlue™ (BioSource International, Nivelles, Belgium) cell viability assay as a suitable indicator of cellular health and viability **(Bannerman *et al*., 2001)**. Briefly, the HGL5 and OVCAR-3 cells were cultured in a 96-well plate (Grainer, Germany). 100 µL of cell suspensions per well (1.5 x 10^4 cells per mL) were seeded and grown overnight in a 5 % CO_2 incubator at 37 °C. After pre-incubation, the cells were grown in the culture medium without (control group) or with punicalagin/pomegranate peel extract at different concentrations. After treatment, 10 µL of AlamarBlue solution was added to each well at the indicated time 4 hours before the endpoint and incubated at 37 °C. The AlamarBlue reduction as a result of multiple metabolic reactions was measured spectrophotometrically. Absorbance was measured at 560 nm and 590 nm by an ELISA microplate reader (Multiskan FC, Thermo Fisher Scientific, Finland). For each experiment, wells containing only the AlamarBlue solution without cells were also prepared and incubated. The fluorescence measured in those was used as a background and subtracted. The results were expressed as the percentage of viable cells. Analyses were performed in three independent experiments with 8 replicates (culture wells per group) per experiment.

ELISA (enzyme-linked immunosorbent assay)

Concentrations of steroid hormones (17β-estradiol and progesterone) after pure punicalagin and pomegranate peel extract treatment secreted by HGL5 were determined spectrophotometrically using ELISA kit (NOVATEC, Dietzenbach, Germany) according to the manufacturer's instructions. Cells were re-seeded in a 24-well culture plate (Grainer, Germany) at a density of 1 x 10^5 cells per mL and incubated in DMEM culture media (control) or with punicalagin/pomegranate peel extract at different concentrations for 24 h. Analyses from three independent experiments were performed with three replicates per experimental group. The level of 17β-estradiol and progesterone were measured at a wave length of 450 nm on an ELISA microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland). Intra-and inter-assay coefficient for 17β-estradiol was set at ≤9% and ≤10%, and for progesterone at ≤4% and ≤9.3%, respectively. The sensitiveness was 8.68 pg/mL for 17β-estradiol and 0.05 ng/mL for progesterone.

Statistical analysis

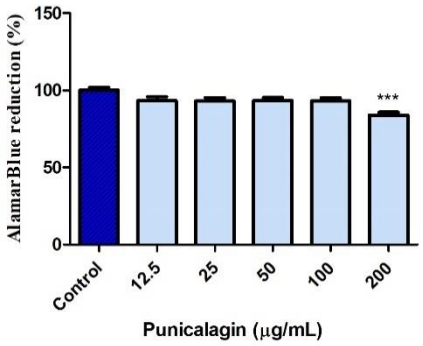
Analyses were performed in at least three independent experiments with replicates per experiment. All data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was carried out using the GraphPad Prism 5 program (version 3.02 for Windows; GraphPad Software, CA, USA). One-way analysis of variance (ANOVA) along with Dunnett's test as a follow-up test to ANOVA was performed as appropriate to determine the statistical significance of differences of the data. The statistical significance was set at probability values of $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of punicalagin on cell viability

To investigate the effects of punicalagin on cell viability in human ovarian cell lines, HGL5 and OVCAR-3 cells were treated with pure punicalagin at different concentrations for 24 h. AlamarBlue cell viability assay was used to measure the number of viable cells. In this *in vitro* study, we observed a significant ($P \leq 0.001$) decrease of viable HGL5 cells after punicalagin treatment only at the highest concentrations of 200 µg/mL. On the other hand, treatment with punicalagin at all the concentrations used in the study did not cause any significant changes ($P > 0.05$) in the viability of human ovarian cancer cells OVCAR-3. The results are shown in Figure 1.

A



B

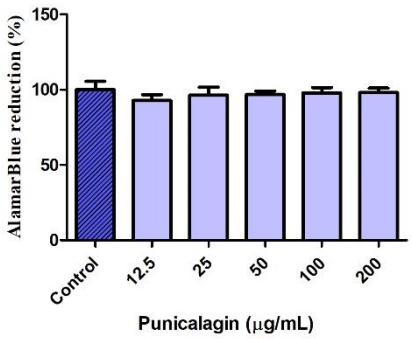
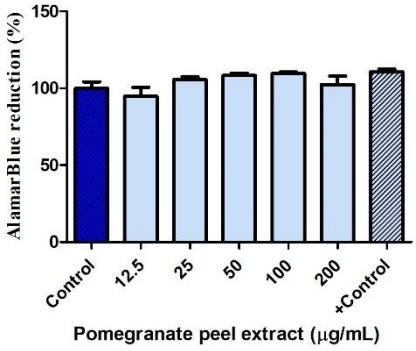


Figure 1 Viability of human ovarian granulosa cells HGL5 (**A**) and human ovarian carcinoma cells OVCAR-3 (**B**) without (control) or with punicalagin treatment (12.5, 25, 50, 100, and 200 µg/mL) for 24 h. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett´s multiple comparison test. The data are expressed as means ± SEM. AlamarBlue.

Effect of pomegranate peel extract on cell viability

To evaluate the effects of pomegranate peel extract on the viability of human ovarian cells HGL5 and OVCAR-3, cells were treated with pomegranate peel extract at different concentrations for 24 h. AlamarBlue cell viability assay was used to measure the number of viable cells. Our data showed a significant decrease (P≤0.001) of cell viability of OVCAR-3 cells in a dose-dependent manner at 25, 50, 100, and 200 µg/mL, but there was no effect (P≥0.05) on healthy ovarian granulosa cells. Pomegranate peel extract used in this study inhibited ovarian cancer cell proliferation *in vitro* but did not affect the viability of HGL5 cells, however, we observed a slight tendency of increase of viable cells at the concentrations 25, 50, and 100 µg/mL. The results are shown in Figure 2.

A



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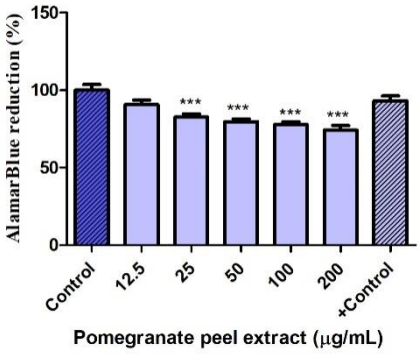
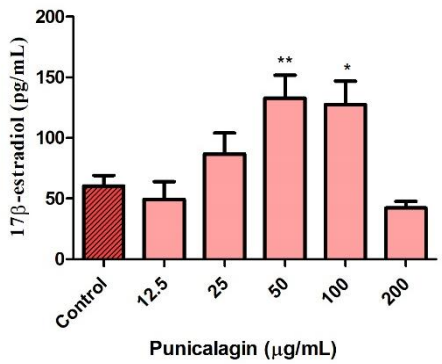


Figure 2 Viability of human ovarian granulosa cells HGL5 (**A**) and human ovarian carcinoma cells OVCAR-3 (**B**) without (control) or with pomegranate peel extract treatment (12.5, 25, 50, 100, and 200 µg/mL) for 24 h. +Control with ethanol in an amount corresponding to the highest used concentration of extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett´s multiple comparison test. The data are expressed as means ± SEM. AlamarBlue.

Effect of punicalagin on the release of steroid hormones

To further evaluate the effects of punicalagin on human ovarian cells *in vitro*, we measured the release of steroid hormones (Figure 3) 17β-estradiol (A) and progesterone (B) by HGL5 cells after punicalagin treatment. Punicalagin could increase the secretion of 17ß-estradiol at the concentration 50 (P≤0.01) and 100 (P≤0.05) µg/mL by HGL5 cells. However, no concentration of punicalagin used in this study affected (P≥0.05) the progesterone secretion in comparison to control.

A



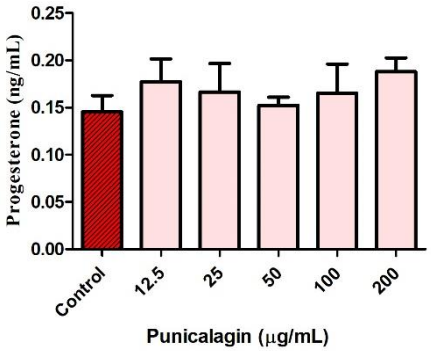


Figure 3 Release of 17β-estradiol (**A**) and progesterone (**B**) by HGL5 cells after treatment with punicalagin (12.5, 25, 50, 100, and 200 μg/mL) for 24 h. Control represents a culture medium without punicalagin. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett´s multiple comparison test. The data are expressed as means ± SEM. ELISA.

Effect of pomegranate peel extract on the release of steroid hormones

Pomegranate peel extract could affect the secretion of 17β-estradiol and progesterone by the HGL5 cells. The results showed a significant increase of the 17ß-estradiol level after pomegranate peel extract supplementation at the concentrations 50 (P≤0.01), 100, and 200 (P≤0.001) μg/mL. Additionally, progesterone secretion was significantly affected after pomegranate peel extract treatment. The level of progesterone was significantly (P≤0.05) decreased at all used concentrations except the highest concentration of 200 μg/mL. The results are shown in Figure 4.

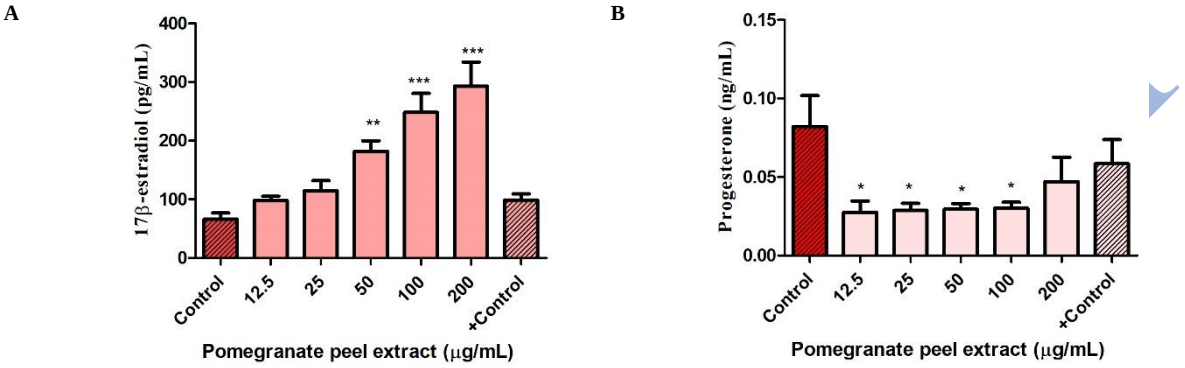


Figure 4 Release of 17β-estradiol (**A**) and progesterone (**B**) by HGL5 cells after treatment with pomegranate peel extract (12.5, 25, 50, 100, and 200 μg/mL) for 24 h. Control represents a culture medium without pomegranate peel extract. +Control with ethanol in an amount corresponding to the highest used concentration of extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett´s multiple comparison test. The data are expressed as means ± SEM. ELISA.

Nowadays, phytonutrients found in fruits and vegetables have been receiving increasing attention for their beneficial impacts on reproductive functions **(Halenar *et al.*, 2016; Packova *et al.*, 2015, 2016; Roychoudhury *et al.*, 2017, 2018, 2020; Michalcova *et al.*, 2019; Baldovská *et al.*, 2019, Kolesárová *et al.*, 2020)**. Investigating the anti-proliferative efficiency of natural biomolecules against cancer has gained increasing momentum for designing potential chemopreventive and chemotherapeutic agents (**Masaud *et al.*, 2014; Zhang *et al.*, 2020**). Natural products with beneficial properties have outstanding anti-cancer activity with high efficiency and minimal side effects, which can induce cell senescence to suppress the occurrence and development of tumours, by inhibiting telomerase activity, triggering DNA damage, and activating or inactivating oncogenes (**Liu *et al.*, 2020).** Application of pomegranate and its extracts has been extensively studied and has so far shown promising results (**Kandys and Kokkinomagoulos, 2020**). The present study discusses the effects of pomegranate peel extract and punicalagin, one of its main phytochemicals on human ovarian cells.

Pomegranate is a rich source of polyphenols and pomegranate extracts are known to possess strong antioxidant properties, including anti-cancer activity on *in vitro* cancer cell models, preclinical laboratory animals, and early phase clinical trials (**Masaud *et al.*, 2014**). In the present study, the biological effects of both pomegranate peel extract and punicalagin on the human granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3 was examined. The experiments were designed to determine the effects of punicalagin and pomegranate peel extract on cell viability and the secretion of selected steroid hormones. The inhibition of proliferation of cancer cells OVCAR-3 by the pomegranate peel extract without a negative impact on healthy granulosa cells was observed. The present findings are consistent with that of a previous study indicating that natural polyphenols effectively inhibit proliferation in ovarian cancer cells (**Zhang *et al.*, 2020**). The results are also in line with other recent studies which demonstrated the anti-proliferative and apoptosis-inducing effect of pomegranate peel extracts and punicalagin on prostate cancer cells (**Adams *et al.*, 2010; Adaramoye *et al.*, 2017**). In accordance with our findings, another previous study showed the cell-specific and dose-dependent anti-proliferative effect of polyphenol-rich pomegranate extract on human ovarian carcinoma cells *in vitro* (**Baldovská *et al.*, 2019**).

By contrast, the present results also showed a significant decrease in the viability ovarian granulosa cells HGL5 after punicalagin supplementation at the highest concentration of 200 μg/mL. Divergent findings have been reported by **Packova *et al.* (2016)**, too, who described the stimulatory effect of punicalagin on the proliferation of porcine ovarian granulosa cells at concentrations 1 μg/mL. Further carefully designed confirmatory studies on the potential roles of pomegranate and its main component punicalagin on cell proliferation are therefore needed to reveal the exact dose-response relationship and the nature of effect.

Many studies have shown that polyphenol punicalagin effectively inhibits cancer cell proliferation (**Wang *et al.*, 2013; Adaramoye *et al.*, 2017; Yao *et al.*, 2017; Cheng *et al.*, 2018; Zhang *et al.*, 2020)**, however, the results from our study demonstrated no significant change in the viability of ovarian cancer cells OVCAR-3. Although the mechanism of action of the pomegranate extract has not been fully established, our results from the tested parameters are in agreement with a study conducted by **Masaud *et al.* (2014)** that the pomegranate extract exerts more anti-cancer effect as compared to any of its individual constituents. This may be due to its multiple substances, which work in tandem to produce its pharmacological activity.

Furthermore, contemporary studies have reported the anti-proliferative and anti-cancer activities of the pomegranate juice, extract, or oil by modulating multiple signaling pathways **(Sharma *et al.*, 2017)**, including the downregulation of Akt/mTOR pathway, and induction of apoptosis by increasing the Bax/Bcl-2 ratio **(Syed *et al.*, 2013)**. Investigations on the possible functional interrelationship between pomegranate´s actions and ovarian cancer revealed that pomegranate fruit juice, ellagic acid and luteolin (phytocomponents of pomegranate) suppressed the proliferation and migration of the ovarian cancer cells and down-regulated the expression of matrix metalloproteinases MMP2 and MMP9, while ellagic acid induced a greater effect than luteolin (**Liu *et al.*, 2017**). In addition, ellagic acid treatment of the human ovarian carcinoma ES-2 and PA-1 cells inhibited cell proliferation with a dose- and time-dependent manner, induced a decrease of cyclins D1 and E levels, and caused an increase of p53 and p21, which led to cell cycle arrest in G1 phase (**Chung *et al.*, 2013**). In order to

further explore the relationship between punicalagin and its biological and anti-cancer activities, researchers observed the induction of cellular senescence via cell cycle arrest and upregulation of p21 (**Wang *et al.*, 2013**). Resveratrol derivative (3,3',4,4'-tetrahydroxy-trans-stilbene) can induce senescence and inhibit cancer cell proliferation, too, which is accompanied by increased DNA damage and ROS production, reduction of DNA damage repair capacity and decrease of activity of enzymatic antioxidants (**Mikula-Pietrasik *et al.*, 2015**). In another study, the mechanism underlying the effect of isoquercitrin on human ovarian carcinoma cells OVCAR-3 was examined. **Michalcova *et al.* (2019**) concluded that the impact of isoquercitrin on ovarian carcinoma cells may be mediated by an antioxidative pathway that involves inhibition of intracellular ROS production, thereby limiting oxidative stress.

Various parts of the fruit, method of extraction, and different solvents can define the phytochemical profile of the pomegranate extracts and their biological activities (**Tamborlin *et al.*, 2020**). Phytochemical characterization of pomegranate peel and seed using ultra-high-performance liquid chromatographic-diode array (UHPLC-DAD) showed a positive correlation between antioxidant capacity and total phenolic content. Additionally, the results showed that pomegranate peel possesses high phenolic (TPC: 224.39 mg GAE/g dw) and flavonoid (TFC: 62.64 mg rutin/g dw) contents and the results also showed that punicalagin-β (216:36 ± 9:94 mg/g) and punicalagin-α (154:94 ± 5:21 mg/g) were the most abundant compounds present in pomegranate peel (**Sabraoui *et al.*, 2020**). In this context, it was previously shown that pomegranate peel could be used for the fortification of functional food products, as well as in health applications due to its higher antioxidant activity.

Pomegranate´s phytonutrients may play an important role as the possible modulator of process of steroidogenesis (**Packova *et al.*, 2015; Baldovská *et al*., 2019**). Polyphenols found in the pomegranate peel and pomegranate juice, especially flavonoids (e.g., flavonols, flavones, and anthocyanidins), and hydrolysable tannins (e.g., ellagitannins and gallotannins) have been hypothesized to reduce breast cancer risk through modulation of sex hormones (**Kapoor *et al.*, 2015**), and pomegranate ellagitannin-derived compounds may modulate estrogen synthesis by inhibition of aromatase activity (**Kim *et al.*, 2002; Adams *et al.*, 2010**).

Similarly, **Modaeinama *et al.* (2015**) examined the anti-cancer properties of a methanolic pomegranate peel extract on different human cancer cells. Interestingly, the most responsive cells to the anti-proliferative effect were breast adenocarcinoma cells MCF-7, whereas ovarian cancer cells SKOV3 were the least responsive cells in comparison to the other monitored cancer cells. Different responsiveness of cells to the anti-proliferative effect of pomegranate could be explained by the hormone-sensitivity of cancer type and pomegranate´s polyphenols could interfere with aromatase activity and so hinder estrogen synthesis which can act as a growth factor of cells.

Clinical studies suggest polyphenolic compounds may exert breast cancer-preventive effects through modulation of endogenous sex hormone levels. Beneficial effects of pomegranate juice consumption on hormonal biomarkers of breast cancer risk, including estradiol, estrone, testosterone, androstenedione, and sex hormone binding globulin (SHBG) was investigated (**Kapoor *et al.*, 2015**). In fact, the proliferative or anti-proliferative effects induced by 17β-estradiol in cancer cells are mediated by two different isoforms of the estrogen receptors, ERα and ERβ. 17β-estradiol via ERα evokes rapid signals to induce proliferation in breast cancer cells, while 17β-estradiol-induces ERβ rapid signaling that inhibits proliferation of colon cancer cells. These contrasting effects could be associated with the molecular complexity of the 17β-estradiol-induced intracellular signaling pathway triggered by the estrogen receptors (**Acconcia and Marino, 2011**). It was shown that ellagic acid present in pomegranate is able to modulate the activity of the estrogen receptor subtypes ERα and ERβ in HeLa cells (**Papoutsi *et al.*, 2005**).

Based on accumulating experimental evidence, there is reason to hypothesize that pomegranate peel extract and punicalagin may alter the secretion of steroid hormones. This *in vitro* study was carried out to reveal pomegranate´s potential in the modulation of secretion of steroid hormones by human ovarian granulosa cells HGL5, too. We evaluated the impact of punicalagin on the secretion of 17β-estradiol and progesterone. Punicalagin treatment at selected concentrations increased 17β-estradiol levels but did not significantly affect progesterone secretion, so punicalagin may be an effector in the process of ovarian steroidogenesis. However, divergent findings have been reported for the response of rabbit ovarian fragments on punicalagin treatment, whereas punicalagin (at 100 mg/mL) increased progesterone levels and the secretion of 17β-estradiol were significantly decreased by the concentration of punicalagin 10 mg/mL (**Packova *et al.*, 2015**). Regarding the effect of pomegranate peel extract supplementation on the tested parameters, we observed significant increase of 17β-estradiol secretion. Our results also demonstrated that progesterone levels were decreased after pomegranate peel extract treatment. The present findings are consistent with previous studies indicating that pomegranate extract can modulate the secretion of steroid hormone 17β-estradiol in human granulosa cells (**Baldovská *et al*., 2019**).

Following previous studies, our data confirm that pomegranate fruit is a unique source of phytoestrogens and divergent cellular effects on HGL5 and OVCAR-3 cells could be associated with the modulative activity of pomegranate peel extract on steroidogenesis. Finally, identification of the mechanisms that are associated with the previously mentioned activities of pomegranate and its compound punicalagin as well as its possible synergistic effects with other phytocompounds are essential for future food applications and further nutraceutical product development with potential health benefits. Therefore, more evidence is needed to clarify the effect of punicalagin and pomegranate peel on human health.

CONCLUSION

In conclusion, phytochemicals present in functional foods offer great hope as an alternative therapy for many disorders. The present study examined the potential modulatory effect of punicalagin and pomegranate peel extract from non-edible parts of *Punica granatum* L. We tested the anti-proliferative effect as well as the effect on the release of steroid hormones by using non-cancerous and cancerous human ovarian cells lines *in vitro*. We suggested that pomegranate peel extract used in this study may contain bioactive compounds, which exert anti-proliferative effects in a cell-dependent manner. It can be indicated that the polyphenol punicalagin and pomegranate peel extract may be a potential endocrine modulator of steroidogenesis in ovarian cells HGL5. Thus, pomegranate peel extract seems to be a better chemopreventive agent in comparison to pure punicalagin, however, further carefully designed studies involving other pomegranate compounds are necessary to reveal their possible anti-cancer activities and the exact dose-response relationships.

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