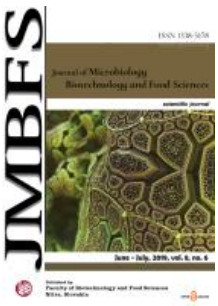

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

POLYPHENOL-RICH POMEGRANATE EXTRACT AS A POTENTIAL MODULATOR OF STEROIDOGENESIS IN HUMAN OVARIAN CELLS

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doi: 10.15414/jmbfs.2019.8.6.1343-1346

ARTICLE INFO

Received 7. 1. 2019
Revised 6. 2. 2019
Accepted 21. 2. 2019
Published 1. 6. 2019

Regular article

OPEN ACCESS

ABSTRACT

Pomegranate represents a rich source of phytocompounds with high medicinal value. Nowadays, many studies have shown that the pomegranate extract also possesses anti-oxidant, anti-inflammatory and anti-proliferative effects on cancer cells, thus leading to increased popularity as a functional food and nutraceuticals. The aim of the study was to determine the biological effect of dry pomegranate extract (at concentrations 5, 10, 20 and 40 µg/ml; for 24h) on the viability of ovarian cells and the secretion of steroid hormones. Cultures of human ovarian granulosa cells (HGL5) and human ovarian carcinoma cells (OVCAR-3) were used such as a model cell system. The metabolic activity was evaluated by AlamarBlue™ assay, the release of steroid hormones was assayed by the ELISA method. Experimental results indicated a significant ($P \leq 0.001$) increase of proliferation in HGL5 cells after the addition of the extract at the concentrations 5, 10 and 20 µg/ml. Moreover, the number of viable OVCAR-3 cells significantly ($P \leq 0.05$; $P \leq 0.01$; $P \leq 0.001$) decreased after the addition of the extract at the concentrations 10, 20 and 40 µg/ml compared to the control. In addition, the secretion of 17ß-estradiol by the HGL5 cells was significantly ($P \leq 0.05$; $P \leq 0.001$) increased at all used concentrations of the extract. Despite to increasing of the 17ß-estradiol secretion, progesterone levels produced by the HGL5 cells were not significantly ($P \geq 0.05$) affected at all used concentrations of the extract. The current study provided experimental evidence that the pomegranate extract might be a promising candidate as a potential modulator of steroidogenesis and as a potential chemoprotective agent.

Keywords: pomegranate, ovarian cells, steroid hormones, cancer

INTRODUCTION

Punica granatum L., commonly known as pomegranate, is a deciduous shrub, native to the Mediterranean region **(Al-Said *et al.*, 2009)**. Nowadays, pomegranate has received attention as a functional food and can be used for dietary supplement or nutraceutical and consumed as such as fresh fruit or in processed juice, jam, wine, and powdered capsules that contain extracts of different pomegranate tissues **(Aqil *et al.*, 2012; Akhtar *et al.*, 2015)**.
Many investigators have reported a free radical scavenging and strong antioxidant properties of pomegranate polyphenols including flavonoids, flavonols, and anthocyanins **(Elfalleh *et al.*, 2011)**. The flavonoids such as luteolin, resveratrol, and quercetin are found in the peel pomegranate extract **(Choi *et al.*, 2006; Viuda-Martos *et al.*, 2010)**. Additionally, pomegranates contain hydrolysable tannins, especially ellagitannins such as punicalagin, punicalin, gallotannin **(Gil *et al.*, 2000; Landete *et al.*, 2011)**, condensed tannins, proancyanidins, anthocyanins **(Zhang *et al.*, 2011)**, organic and phenolic acids, including ellagic and gallic acid (**Mousavinejad *et al.*, 2009**), sterols, triterpenoids, fatty acids, triglycerides, and alkaloids **(Seeram *et al.*, 2005)**. Various parts of the pomegranate fruit have been shown to exert multiple beneficial effects on human health. Clinical and preclinical studies have determined that pomegranates have anti-oxidant (**Singh *et al.*, 2014; Zhang *et al.*, 2011**), anti-inflammatory **(Gonzalez-Trujano *et al.*, 2015)**, anti-bacterial **(Viladomiu *et al.*, 2013)**, anti-cancer **(Syed *et al.*, 2013; Sharma *et al.*, 2017)**, anti-obesity **(Al-Muammar *et al.*, 2012)**, and neuroprotective **(Yuan *et al.*, 2016)** activities.
Some plants, including *Punica granatum* L., contain phytoestrogens. Punic acid, kaempferol and β-sitosterol present in pomegranate have shown phytoestrogenic activity **(Choi *et al.*, 2006)**. Phytoestrogens are structurally similar to steroid hormone 17β-estradiol and compete with the endogenous hormone for binding to estrogen receptor, thus reducing the hormonal effect of endogenous estrogens **(Papoutsi *et al.*, 2005)**.
The ovaries are responsible for the production of sex steroids, various growth factors, transcription factors and cytokines **(Kolesarova *et al.*, 2015)**. The steroid hormones are the best-known and best-characterized secretory products of the ovary **(Schams and Berischa, 2002)**. The influence of steroid hormones, including 17β-estradiol, progesterone, and others on ovarian functions and female fertility is complex and still requires elucidation. Understanding the mechanisms which regulate ovarian steroidogenesis at the molecular and cellular level requires readily available cells for *in vitro* studies **(Havelock, 2004)**. The Immortalized human granulosa cell line HGL5 has been previously described in detail **(Rainey *et al.*, 1994)**. The HGL5 cell line exhibits qualities consistent with primary ovarian granulosa cells, includes the ability to produce progesterone and 17β-estradiol, and offer an optimal tumor-like system for studies focused on gonadotropin-dependent proliferation, cell survival and apoptosis **(Patel *et al.*, 2009)**. Human ovarian epithelial carcinoma cell line, NIH: OVCAR-3 is an appropriate model system in which to study drug resistance in ovarian cancer, the presence of hormone receptors and has been previously described in detail **(Hamilton *et al.*, 1983)**. Therefore, selected ovarian cell lines HGL5 and OVCAR-3 can serve as useful models for studying signalling pathways and regulation of steroid biosynthesis.
In this *in vitro* study, the effect of dry pomegranate extract on human ovarian cells, using HGL5 and OVCAR-3 cells was investigated. The objective was to examine the viability of human ovarian cells and the secretion of selected steroid hormones after the addition a number of the pomegranate extract concentrations (5, 10, 20 and 40 µg/ml).

MATERIAL AND METHODS

Cell lines

The human ovarian granulosa cell line HGL5 (ABM®, BC, Canada) were cultured in Dulbecco's modification of 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 until 80–90 % confluent. The cells were grown in a standard T75 cell culture flask (Corning Life Sciences, NY, USA) to 80–90 % confluence.

The human ovarian carcinoma cell line, NIH: OVCAR-3 was obtained from the American Type Culture Collection (ATCC®, VA, USA). The cells were cultured in culture medium RPMI1640 (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 cells were grown in a standard T75 cell culture flask (Corning Life Sciences, NY, USA) to 80-90 % confluence.

Pomegranate extract treatment

Pomegranate extract used in this study get from commercially purchased capsules from Spain, which contain dry extract of *Punica granatum*. Total polyphenols in the pomegranate powder present not than less 50 % and punicalagins (α and β) present even 30 % of the content. Prior to the experiments, pomegranate extract powder was dissolved in culture medium and diluted to the desired concentrations. Depending on the pomegranate extract treatment, the cells were cultured in plates without (control group) or with pomegranate extract at concentrations 5, 10, 20 and 40 µg/mL for 24 h.

Cell viability assay

Cell viability was evaluated using AlamarBlue™ (BioSource International, Nivelles, Belgium) 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) per 100 µL at the density of 1.5 x 10^4 cells per well and grown overnight in a 5 % CO_2 incubator at 37 °C. After pre-incubation, the cells were grown in culture for 24 hours without (control group) or with pomegranate extract (5, 10, 20 and 40 µg/mL). The AlamarBlue solution was added to each well 4 hours before the endpoint at a volume of 10 µL. The AlamarBlue reduction as a result of multiple metabolic reactions was measured spectrophotometrically by recording the absorbance at 560 nm and 590 nm using an ELISA microplate reader (Multiskan FC, ThermoFisher 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 replicates per experiment.

ELISA (enzyme-linked immunosorbent assay)

Concentrations of secreted steroid hormones after supplementation by pomegranate extract were determined using ELISA kit (NOVATEC, Dietzenbach, Germany) according to the manufacturer's instructions. Cells HGL5 were re-seeded in a 24-well culture plate (Grainer, Germany) at a density of 1 x 10^5 cells per well and incubated in DMEM culture media (control) or with pomegranate extract (at concentrations 5, 10, 20 and 40 µg/mL) for 24 h. The release of 17β-estradiol and progesterone was measured spectrophotometrically according to the manufacturer's instructions at a wavelength 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%, for progesterone was set as ≤ 4% and ≤ 9.3%. The sensitiveness was 8.68 pg/mL for 17β-estradiol and 0.05 ng/mL for progesterone. The results were averaged over 3 different independent experiments with replicates per experiment.

Statistical analysis

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). Experiments were repeated three times in duplicate for experiments. One-way ANOVA along with Dunnett's test as a follow-up test to ANOVA was performed as appropriate to determine the statistical significance. The statistical significance was established at P≤0.05, P≤0.01, P≤0.001.

RESULTS AND DISCUSSION

Effects of pomegranate extract on cell viability

In this *in vitro* study, we observed a significant (P≤0.001) increase of viable HGL5 cells after treatment of pomegranate extract at the concentrations 5; 10 and 20 µg/ml. Moreover, our results indicated significantly (P≤0.05; P≤0.01; P≤0.001) inhibited the proliferation of OVCAR-3 cells in a dose-dependent manner after pomegranate extract application at the concentrations 10; 20 and 40 µg/ml. The results are shown in the figure 1.

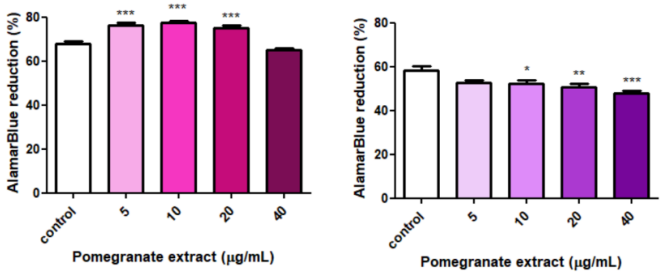

Figure 1 The viability of human ovarian granulosa cell line (**A**) and human ovarian carcinoma cell line (**B**) without (control) or with pomegranate extract treatment (5, 10, 20 and 40 µg/mL) for 24 h. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett´s multiple comparison test. The data are expressed as means ± SEM. AlamarBlue.

Effects of pomegranate extract on the release of steroid hormones

The current study suggests that pomegranate extract could have an impact on the secretion of steroid hormones – 17β-estradiol and progesterone by the cells. The results showed a significant (P≤0.05; P≤0.001) increase of the 17ß-estradiol secretion by pomegranate extract treatment at the concentrations 5; 10; 20 and 40 µg/ml in comparison to control. On the other hand, progesterone levels were not significantly (P≥0.05) affected at all used concentrations in comparison to control after addition of pomegranate extract. The results are shown in the figure 2.

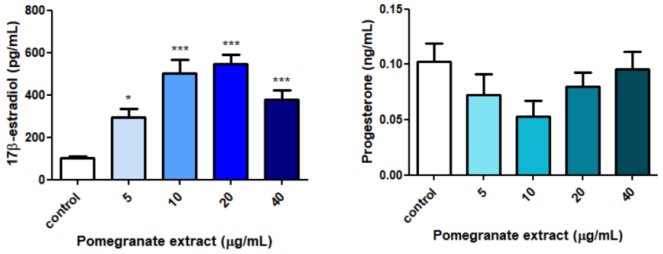

Figure 2 The release of 17β-estradiol (**A**) and progesterone (**B**) by HGL5 cells after treatment with pomegranate extract (5, 10, 20 and 40 µg/mL) for 24 h. Control represents culture medium without pomegranate extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett´s multiple comparison test. The data are expressed as means ± SEM. ELISA.

Nowadays, natural biomolecules and their impact on reproductive functions have attracted a huge scientific attention **(Kolesarova *et al*., 2011; Halenar *et al*., 2016; Packova *et al*., 2016; Roychoudhury *et al*., 2018)**. Naturally-occurring phytocompounds from dietary fruits and vegetables have received public interest for the prevention and treatment of various diseases **(Modaeinama *et al*., 2015; Sharma *et al*., 2017)**.

The quest for effective and safe chemotherapeutic agents is urgently necessary for a view of the fact that many anticancer drugs elicit harmful side effects on normal cells. The current experiments were designed to determine the biological effects of rich-polyphenol pomegranate extract on the viability of human ovarian cells. The stimulation of proliferation of healthy human granulosa cells HGL5 by the pomegranate extract *in vitro* was observed.

The beneficial properties associated with punicalagin as a major phytocompound of pomegranate, described **Packova *et al.* (2016)**. In previous study, the pro-proliferative effect of punicalagin on porcine ovarian granulosa cells *in vitro* was examined. Further studies of the potential roles of pomegranate in proliferation are therefore needed.

Many reports have shown that the pomegranate as well as juice, extract or oil exert antiproliferative, anti-inflammatory, and anti-cancer properties by modulating multiple signalling pathways **(Sharma *et al*., 2017)**. In current studies, antioxidants have been examined due to their protective properties against free radical-induced damage. Clinical and experimental studies proved the relationship between a polyphenol-rich diet and reduced risk of degenerative diseases **(Vauzour *et al*., 2010; Viuda-Martos *et al*., 2010)**.

Ovarian cancer is one of the most common malignancies in the female reproductive system with high mortality rates worldwide **(Liu *et al*., 2017)**. In this study, the cell-specific and dose-dependent antiproliferative effect of pomegranate extract as a promising chemo-preventive agent was examined. The polyphenol-rich pomegranate extract inhibited the proliferation of human ovarian

carcinoma cells OVCAR-3. In accordance with findings the present study, the results conclusively demonstrated the association between dietary flavonoids intake and ovarian cancer risk **(McCann *et al.*, 2003; Chang *et al.*, 2007)**.

Pomegranate polyphenols are potent antioxidants which have been shown both *in vitro* and *in vivo* to inhibit the growth of several forms of cancer **(Toi *et al.*, 2003; Elfalleh *et al.*, 2011; Modaeinama *et al.*, 2015)**. Many investigations have established tremendous potential of pomegranate as an anticancer agent against various cancer cells including breast **(Kim *et al.*, 2002; Adams *et al.*, 2010)**, lung **(Khan *et al.*, 2007)**, colon **(Larrosa *et al.*, 2006)**, skin **(Hora *et al.*, 2003)**, prostate **(Hong *et al.*, 2008; Seidi *et al.*, 2016)** and others. Numerous studies have reported, that pomegranate extracts may affect proliferation by downregulating Akt/mTOR pathways and may induce apoptosis of cancer cells by increasing the Bax/Bcl-2 ratio **(Syed *et al.*, 2013; Sharma *et al.*, 2017)**.

Recently, punicalagin and ellagic acid, present in pomegranate, was described as a possible effector of the cell cycle and have a proapoptotic effect **(Gil *et al.*, 2000)**. The potent antioxidant pronounced antiproliferative and apoptosis-inducing effect of punicalagin in prostate cancer cells *in vitro* was observed **(Adaramoye *et al.*, 2017)**.

In vitro human cell line models are available for a variety of malignancies, serving as suitable platforms for exploring antiproliferative and cytotoxic effects of natural products. Direct cytotoxic effects might be apoptosis or necrosis (or both) mediated, with a number of mechanisms leading to cell death, changes in proliferation patterns and effects on cell differentiation (**Choi *et al.*, 2006**). Human ovarian granulosa cells HGL5 maintain key steroidogenic pathways, making them an attractive model to study mechanisms of steroid biosynthesis **(Patel *et al.*, 2009)**. Therefore, we monitor also secretion of steroid hormones by the cells after pomegranate extract treatment. Interestingly, the supplementation by pomegranate extract at selected concentrations increased the secretion of 17β-estradiol by HGL5 cells but did not significantly affected progesterone levels. In according to previous studies **(Papoutsi *et al.*, 2005; Tran *et al.* 2010)**, our data suggest that pomegranate is an excellent source of phytoestrogens.

The focus of major research and clinical attention is concentrated on phytoestrogens due to its effectiveness in the prevention and treatment of menopausal symptoms, osteoporosis, cancer and other diseases **(Liu *et al.*, 2001; Choi *et al.*, 2006)**. In contrast to the use of synthetic estrogens, the functional foods containing elevated amounts of phytoestrogens have been recommended for the prevention of breast cancer. One such food is pomegranate fruits that contain phytocompounds with hormone-like activity **(Kim *et al.*, 2002)**.

The main polyphenolic compounds present in the pomegranate fruits of are ellagitannins, such as punicalagin (α and β), and flavonoids, such as anthocyanidins, catechins, and flavonols **(Gonzalez-Trujano *et al.*, 2015)** and possess estrogenic activity on *in vitro* assays **(Larossa *et al.*, 2006; Landete *et al.*, 2011)**. **Papoutsi *et al.* (2005)** investigated the ability of ellagic acid to influence the activity of the estrogen receptor subtypes ERα and ERβ in HeLa cells.

The effect of the most abundant ellagitannin in pomegranate – punicalagin, on steroidogenesis was examined and the results showed that punicalagin may affect the secretion of 17β-estradiol as the final product of the pathway **(Packova *et al.*, 2015)**. However, the synergistic action of the pomegranate biosubstances appears to be superior when in comparison to individual components **(Viladomiu *et al.*, 2013)**.

Similarly, **Ming *et al.* (2014)** reported the impact of pomegranate extract on steroidogenesis in prostate cancer cell line and prostate cancer mouse model and suggested that androgen biosynthesis might favour the backdoor pathway over the classical Δ4 and Δ5 pathways.

Finally, phytocompounds with phytoestrogenic activity from pomegranate extract could have a potential role as the possible effector in the process of steroidogenesis. The results from previous studies support the fact that pomegranate is indeed a unique fruit and potent source of biologically active phytochemicals with beneficial properties.

CONCLUSION

In conclusion, the pomegranate extracts may be used as a source of health-promoting phytocompounds for developing new functional foods. The beneficial effect of pomegranate extract on the viability of HGL5 cells and antiproliferative effect on the cancer cells OVCAR-3 was studied and quantified. Thus, it can be indicated that pomegranate fruit may contain the bioactive compounds which possess both antiproliferative effect and cell proliferative stimulator in cell-dependent manner. Additionally, rich-polyphenol pomegranate extract may be a potential endocrine modulator of steroidogenesis in ovarian cells. However, further research is essential to understanding the therapeutic potential of pomegranate and its mechanisms of action.

Acknowledgements: This work was supported by the Ministry of Education, Science, Research and Sport of the Slovak Republic projects VEGA 1/0039/16, APVV- 15-0543, APVV-16-0289, The Excellent scientific team "Center of Animal Reproduction (CeRA)", Tatra bank Foundation 2018, and European Community under project number 26220220180 for Building Research Centre "AgroBioTech".

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