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SHORT COMMUNICATION



Anti-proliferative effect of pomegranate peel extracts on bovine peripheral blood mononuclear cells (PBMCs)

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ABSTRACT

Pomegranate peel extracts prepared in our laboratories from a waste of juice fruit processing were tested on bovine peripheral blood mononuclear cells to evaluate the effects on viability, oxidative stress and proliferation. The (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay pointed out that the extracts were not cytotoxic at the tested concentrations (0.1, 1.0, and 10 µg/mL). A moderate protective effect against Reactive Oxygen Species production induced by hydrogen peroxide or lipopolysaccharide and a significant anti-proliferative activity against proliferation induced by concanavalin A were observed on cell lines treated with the extracts at 10 µg/mL. Based on these results, pomegranate peel extracts seem promising as feed supplement for dairy cattle, in particular around calving, when the animals are subjected to an increase of the metabolic activity, responsible for oxidative stress and diseases. However, *in vivo* studies are needed to investigate the stability of the extracts across the bovine gastrointestinal tract barrier.

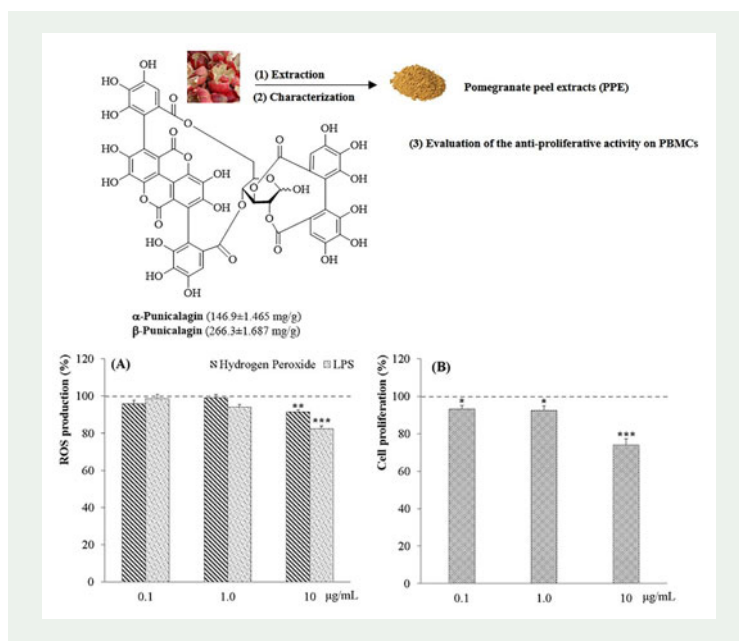
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KEYWORDS

Pomegranate peel extracts; agro-industrial by-products; polyphenols; peripheral blood mononuclear cells; anti-proliferative effect; circular economy



1. Introduction

Plants are a precious source of valuable compounds exhibiting biological and pharmacological effects as antioxidant, antimicrobial and anti-inflammatory activities. For these relevant properties, plant extracts have been used in the past against numerous disorders and diseases, and currently they are object of intense studies to validate their potential as therapeutic agents in the promotion of health and disease prevention (D'Eliseo et al. 2019; Frezza et al. 2019; Mastrogiovanni et al. 2019).

Polyphenols are secondary metabolites variously distributed in the plant kingdom “derived from the shikimate/phenylpropanoid and/or the polyketide pathway, featuring more than one phenolic unit and deprived of nitrogen-based functions” (Yoshida et al. 2016). They exhibit many beneficial effects for human health benefits including the antioxidant, anti-inflammatory and anticancer activity (Barontini et al. 2014; Bernini et al. 2015, 2018) and can be extracted from plants, fruits, and vegetables. When they were recovered from agro-industrial waste and by-products using green extractive technologies, a virtuous process of reuse and valorization process with economic and environmental benefits can be realized according to the “circular economy” strategy (Bianco et al. 2006; Romani et al. 2016; Bernini et al. 2017).

Pomegranate (*Punica granatum* L.) is a fruit crop brought in the Mediterranean area by the Phoenicians over 4000 years ago, which over the time has had a strong increase. Fruits, peel and seeds are considerable sources of polyphenols as hydroxycinnamic acids, hydroxybenzoic acids, flavonoids and hydrolyzable tannins (Singh et al. 2018; Russo et al. 2019).

Despite the wide number of *in vitro* and *in vivo* studies of pomegranate extracts to demonstrate the beneficial effects on human health, little is reported in the veterinary

field. Based on this lack of literature, we recently planned to investigate the role of natural extracts to prevent the onset of the oxidative stress on dairy cattle during the calving period, when the animals are subject to an increase of the metabolic activity (Sordillo and Aitken 2009) and to endocrine variations, which can produce immunosuppression and serious diseases (Lacetera 2012).

In this context, we recently published a study on the activity of pomegranate peel extracts (PPE), rich of punicalagins on the oxidative stress and inflammatory status induced on bovine mammary epithelial cells, BME-UV1 (Mastrogiovanni et al. 2018). The experimental results have showed that, at non-cytotoxic concentrations (0.1, 1.0, and 10 $\mu\text{g/mL}$), PPE produced a reduction of the oxidative stress and at 10 $\mu\text{g/mL}$ of the inflammatory status on BME-UV1 (Mastrogiovanni et al. 2018).

Based on these preliminary data, as a part of our research focused on the evaluation of PPE as food supplement for dairy cattle, in this study we investigated the viability, the protective effect against Reactive Oxygen Species (ROS) production induced by hydrogen peroxide (H_2O_2) or lipopolysaccharide (LPS) and the anti-proliferative effect on bovine peripheral blood mononuclear cells (PBMCs) induced by concanavalin A (ConA). To the best of our knowledge, this is the first study reporting the effects of PPE on bovine PBMCs.

2. Results and discussion

PEE were obtained by a green extraction procedure based on the use of water (Imperatori et al. 2018). As depicted in Figure S1, the HPLC analysis evidenced that PPE resulted rich of punicalagin (α - and β -isomer: 31.5 and 57.2% w/w, respectively) together to minor components as α - and β -punicalin (3.10% w/w), granatin B (1.48% w/w), ellagic acid and derivatives (6.25% w/w), gallic acid (0.38% w/w).

Then, the cytotoxicity of PPE on PBMCs was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay at the concentrations previously tested by us on BME-UV1 (0.1, 1.0, and 10 $\mu\text{g/mL}$). The experimental data graphed in Figure 1 showed that any concentration resulted cytotoxic on PBMCs.

Therefore, the effects of PPE at these concentrations were evaluated on PBMCs after ROS production induced by H_2O_2 or LPS. After 48 h of treatment, PPE at 10 $\mu\text{g/mL}$ showed a protective effect against ROS production induced by H_2O_2 (−8.6%) or LPS (−17.5%); conversely, at the lowest concentrations, PPE did not show significant differences compared to the control (Figure 2A).

Finally, we investigated the effect of proliferation in PBMCs induced by ConA and treated with 0.1, 1.0, and 10 $\mu\text{g/mL}$ of PPE. The Figure 2B evidenced a significant decrease of proliferation; in particular, PPE at 10 $\mu\text{g/mL}$ induced a reduction of 26%. In accordance with the literature data, this activity could be related to punicalagin found in PPE (88.8%); in fact, it is described that this hydrolyzable tannin is able to downregulate the IL-2 expression and cell proliferation via inhibition of NFAT activation showing a potent immune-suppressive activity (Lee et al. 2008).

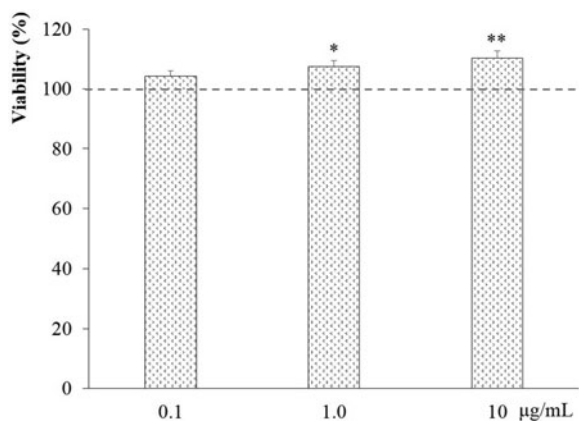


Figure 1. Cells viability after 48 h of treatment with PPE (0.1, 1.0, and 10 µg/mL). Data (mean ± standard error) are expressed as relative percent response respect to control level; cells non-treated with PPE are represented by horizontal dashed line. (* $p < 0.05$; ** $p < 0.01$).

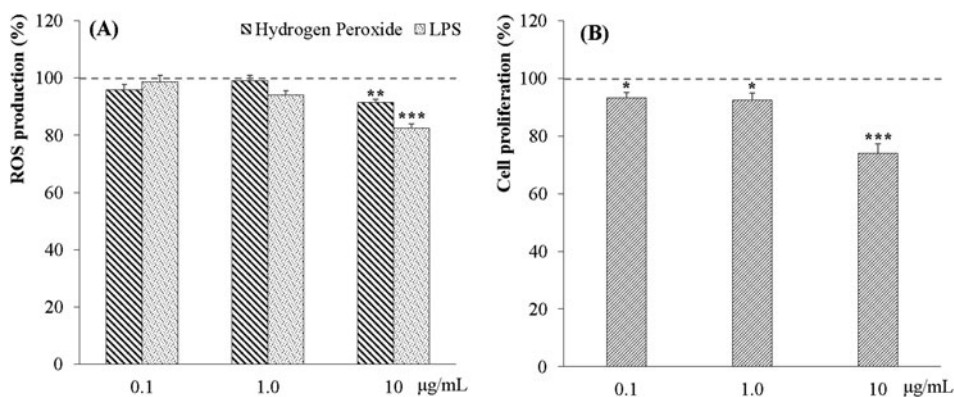


Figure 2. (A) ROS production after 48 h of treatment with PPE (0.1, 1.0, 10 µg/mL) and 3 h of induction with H_2O_2 and LPS. (B) Cell proliferation after induction by ConA and co-treatment with PPE (0.1, 1.0, and 10 µg/mL). Data (mean ± standard error) are expressed as relative percent response respect to control level. (A) Cells treated with H_2O_2 or LPS; (B) cells treated with ConA are represented by horizontal dashed line. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3. Conclusions

This study has evidenced for the first time that PPE rich of punicalagins, obtained from a by-product of the juice industry, exhibited biological effects on bovine PBMCs, reducing ROS production induced by H_2O_2 or LPS and proliferation induced by ConA. The protective effect against ROS production was modest while the anti-proliferative activity induced by ConA was significant. These data, combined to those obtained in our previous study (Mastrogiovanni et al. 2018), make PPE a promising ingredient of feed supplement in dairy cattle to prevent and counteract various immune system derived pathologies, in particular during the calving period. However, *in vivo* studies needed to investigate the bioavailability of PPE in relation to the oral route of administration and the stability across the bovine gastrointestinal tract barrier.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary Material

Anti-proliferative effect of pomegranate peel extracts on bovine peripheral blood mononuclear cells (PBMCs)

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Abstract. Pomegranate peel extracts prepared in our laboratories from a waste of juice fruit processing were tested on bovine peripheral blood mononuclear cells to evaluate the effects on viability, oxidative stress and proliferation. The (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay pointed out that the extracts were not cytotoxic at the tested concentrations (0.1, 1.0 and 10 $\mu\text{g/mL}$). A moderate protective effect against Reactive Oxygen Species production induced by hydrogen peroxide or lipopolysaccharide and a significant anti-proliferative activity against proliferation induced by concanavalin A were observed on cell lines treated with the extracts at 10 $\mu\text{g/mL}$. Based on these results, pomegranate peel extracts seem promising as feed supplement for dairy cattle, in particular around calving, when the animals are subjected to an increase of the metabolic activity, responsible for oxidative stress and diseases. However, *in vivo* studies are needed to investigate the stability of the extracts across the bovine gastrointestinal tract barrier.

Keywords: pomegranate peel extracts; agro-industrial by-products; polyphenols; peripheral blood mononuclear cells; anti-proliferative effect; circular economy.

Experimental

Chemicals

All reagents were purchased from Sigma-Aldrich (Milan, Italy).

Extract preparation and chemical characterization

Pomegranate fruits (“Wonderful” variety) were collected in Grosseto (Tuscany, Italy; Latitude: 42°45'46"N; Longitude: 11°06'33"E) in September 2017. Standardized peel extracts were prepared as briefly described (Imperatori et al., 2018; Mastrogiovanni et al., 2018). Fresh pomegranate peels were frozen with liquid nitrogen and crumbled in a mortar to obtain a powder, which was treated with ultrapure water (w/v=1/12) (MilliQ system waters Co., Milford, MA, USA) at 100 °C for 1h. After filtration, water was removed by lyophilization (LYOVAC GT 2) and the extracts (PPE) were stored in a laboratory dryer in amber glass bottles until the use.

A sample of PPE was analyzed by High Pressure Liquid Chromatography (HPLC/DAD/ESI-MS) and Nuclear Magnetic Resonance (NMR). The HPLC/DAD/ESI-MS analyses were carried out using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies), a Luna C18 column 250×4.60 mm, 5 µm (Phenomenex) according to already reported in the literature (Romani et al., 2012). The NMR spectra were recorded using a 400 MHz Nuclear Magnetic Resonance spectrometer Advance III (Bruker, Germany). Analytical data were detailed in our previous papers (Imperatori et al., 2018; Mastrogiovanni et al., 2018).

Blood collection and PBMCs separation

Eight mid pregnant Holstein heifers (between 3 and 6 months of pregnancy) were used as blood donors. Blood was collected by a vacutainer system (anticoagulant: sodium heparin 10 IU/mL) from jugular vein.

After collection, blood samples were transferred to the laboratory using a portable refrigerator. PBMCs were isolated as previously described (Lacetera et al., 2002). Briefly, PBS (Sigma, St. Louis, MO) was added to all samples. Then, they were layered on Ficoll-Paque PLUS medium (APB, Milan, Italy) and centrifuged at 600 x g and 18°C for 45 min. The mononuclear cell band was recovered through glass pipettes and washed twice in PBS. The red blood cells still present were removed with sterile-

distilled water causing hypotonic shock. The viability (typically over 90%) was determined with trypan blue exclusion method, through automated cell counter (Countess™ - Invitrogen™). PBMCs were suspended at 1×10^6 cells/mL of concentration and were cultured in a culture medium composed by RPMI 1640 medium containing 25 mM HEPES + 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 U of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B/mL.

Cytotoxicity evaluation

The cytotoxicity of PPE at 0.1, 1.0, and 10 µg/mL was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, after 48 h. Cells were plated at 2×10^5 cells/well of concentration into 96-well plates (Sarstedt AG & Co.) contained PPE dilutions or the culture medium (control) and later were kept at 39°C humidified 5% CO₂ incubator. After 48 h, plates were centrifuged at 300 x g for 10 min then treatments were removed and replaced with 100 µL of solution composed by the medium and MTT to a final concentration of 0.5 mg/mL. Then, after 4 h, plates were again centrifuged at 300 x g for 10 min; supernatants were discarded and replaced with 100 µL of dimethyl sulfoxide; plates were additionally incubated for 45 min and finally the absorbance at 595 nm was recorded using a microplates reader (Tecan Sunrise™).

ROS evaluation

ROS production was induced in PBMCs treating cells with PPE and adding hydrogen peroxide (H₂O₂) or lipopolysaccharide (LPS) as direct and indirect oxidant agent, respectively.²² Experimentally, 2×10^5 cells/well were plated in 96-well black plates (Lumox®, Sarstedt AG & Co.) useful to fluorescence reading containing non-cytotoxic PPE concentrations (0.1, 1.0, 10 µg/mL) or the culture medium (control). After 48 h, in each well H₂O₂ (50 µM of final concentration) or LPS (20 µM of final concentration) were added, then microplates were repositioned in incubator at 39°C. Three hours later, plates were centrifuged at 300 x g for 10 min, all supernatants were discarded and replaced by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) 20 µM in PBS and repositioned in incubator. After 40 minutes, the absorbance at 485 nm (excitation) and 535 (emission) was measured using a microplate reader (Ultimode Detector DTX 880, Beckman Coulter Inc.).

Proliferation evaluation

The effect of PPE on the PBMCs proliferation induced by concanavalin A (ConA) was evaluated using a commercial ELISA kit (Cell Proliferation ELISA, BrdU - Roche) based on the determination of 5-bromo-2-deoxyuridine (5-BrdU) incorporated in the DNA of cells in proliferation status. Experimentally, 2×10^5 cells/well were plated in 96-well plates (Sarstedt AG & Co.) containing non-cytotoxic concentrations of PPE (0.1, 1.0, 10 $\mu\text{g/mL}$) or the culture medium (control). After addition of ConA, plates were kept in incubator for 48 h. Then, 5-BrdU was added in each well and plates were incubated for 48 h at 39°C. Finally, following the manufacturer's instructions plates were read at 450 nm by microplate reader (Tecan Sunrise™) and data were recorded.

Statistical analysis

All biological assays described were carried out in triplicate and data were converted in percentages compared to the control. All data were analyzed by ANOVA using Statistica 10 Software package (Stat Soft, Inc., USA) and are reported as mean \pm standard error. The significant of the differences was assessed by the Fisher's Least Square Difference (LSD) test and significance was declared at $p < 0.05$.

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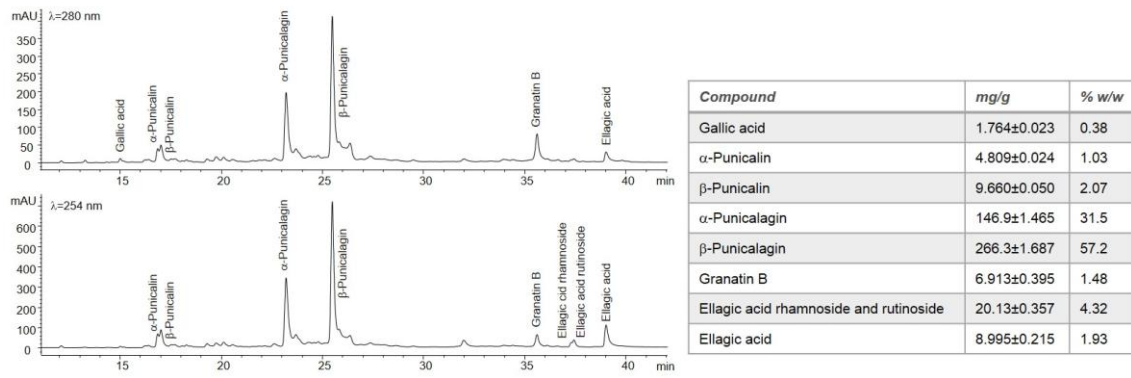


Figure S1. Polyphenolic composition of PPE (Imperatori et al., 2018; Mastrogiovanni et al., 2018)