

Natural Product Research

Formerly Natural Product Letters

ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: <http://www.tandfonline.com/loi/gnpl20>


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To cite this article: Fabio Mastrogiovanni, Roberta Bernini, Loredana Basiricò, Umberto Bernabucci, Margherita Campo, Annalisa Romani, Luca Santi & Nicola Lacetera (2018): Antioxidant and anti-inflammatory effects of pomegranate peel extracts on bovine mammary epithelial cells BME-UV1, *Natural Product Research*, DOI: [10.1080/14786419.2018.1508149](https://doi.org/10.1080/14786419.2018.1508149)

To link to this article: <https://doi.org/10.1080/14786419.2018.1508149>

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 Published online: 02 Oct 2018.

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Antioxidant and anti-inflammatory effects of pomegranate peel extracts on bovine mammary epithelial cells BME-UV1

Fabio Mastrogiovanni^a, Roberta Bernini^a, Loredana Basiricò^a, Umberto Bernabucci^a , Margherita Campo^b, Annalisa Romani^b, Luca Santi^a and Nicola Lacetera^a 

^aDepartment of Agricultural and Forestry Sciences (DAFNE), University of Tuscia, Viterbo, Italy;

^bDepartment of Statistics, Computing, Applications "Giuseppe Parenti" DISIA, Phytolab, University of Florence, Florence, Italy

ABSTRACT

Pomegranate peel extracts (PPE) were tested for the first time on BME-UV1, a valid cellular model to study the bovine mammary epithelial metabolism, to evaluate the effects on the oxidative stress and inflammatory status. Based on the statistical analysis of MTT data, PPE at 0.1, 1.0 and 10 $\mu\text{g/mL}$ resulted not cytotoxic after 24 h, 48 h and 7 days of treatment. At the same concentrations, PPE induced a reduction of ROS production elicited by the addition of hydrogen peroxide or lipopolysaccharide evidencing an antioxidant effect confirmed also by a decrease of malondialdehyde. At 10 $\mu\text{g/mL}$, PPE reduced pro-inflammatory cytokines expressions showing an anti-inflammatory effect on BME-UV1 treated with lipopolysaccharide. Although *in vivo* experiments are necessary, the results of this study are promising for future applications of PPE as feed supplement for dairy cattle, in particular around calving, when the animals are more subject to oxidative stress and inflammatory diseases.

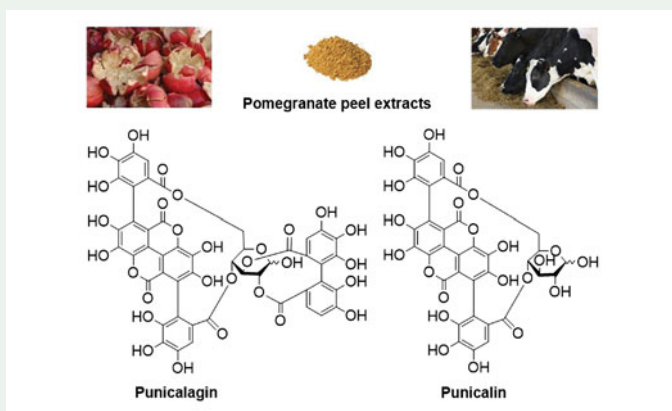
ARTICLE HISTORY

Received 19 June 2018


Accepted 2 August 2018

KEYWORDS

pomegranate peel extracts; hydrolyzable tannins; punicalagin; antioxidant effect; anti-inflammatory effect; bovine mammary epithelial cells; BME-UV1



CONTACT Prof. Roberta Bernini  berninir@unitus.it  Department of Agricultural and Forestry Sciences, University of Tuscia, Via San Camillo de Lellis, 01100 Viterbo, Italy

 Supplemental data for this article can be accessed <https://doi.org/10.1080/14786419.2018.1508149>.

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1. Introduction

Pomegranate (*Punica granatum* L.) peel, a by-product of fruits processing, e.g. juice production, is a good source of bioactive phenolic compounds such as hydrolyzable tannins. Recent studies reported on the biological activity (Imperatori et al., 2018) and health benefits of both pomegranate peel extracts (PPE) and their components including the antioxidant and anti-inflammatory activities (El-Missiry, et al., 2015; Omar et al., 2016; Seok et al., 2018; Singh et al., 2018) which are relevant to prevent several serious diseases. However, to the best of our knowledge, the effects of PPE on bovine mammary cells have never been investigated so far.

Around calving, dairy cattle are subject to an intense metabolic activity, which could lead to an overproduction of Reactive Oxygen Species (ROS), harmful species responsible for the onset of the oxidative stress (Bernabucci et al., 2005). In addition, the animals are subject to endocrine and metabolic variations, which can cause inflammatory diseases (Lacetera et al., 2012) with both negative consequences on their health and economic losses for the farmers. In this scenario, natural compounds with antioxidant and anti-inflammatory activities are indeed promising candidates as feed supplement for dairy cattle in order to prevent and/or counteract periparturient related problems. This choice becomes even more attractive if bioactive compounds are recovered from agro-industrial by-products according to the recent “circular economy” strategy, which encourages the reuse and valorization of waste (Bernini et. al., 2018). Based on these considerations, the present work reported for the first time the evaluation of the activity of a standardized PPE on the oxidative stress and inflammatory status induced in bovine mammary epithelial cells BME-UV1.

2. Results and Discussion

PPE were obtained from fruits peel by an extractive process recently described by our group (Imperatori et al., 2018). The extract, characterized by HPLC/DAD/ESI-MS and ¹H-NMR analysis, resulted rich in hydrolyzable tannins, such as α - and β -punicalagin, α - and β -punicalin; minor components were granatin B, gallic acid, ellagic acid and derivatives (Figure S1).

Cell viability was measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay on BME-UV1, a valid cellular model to study the bovine mammary epithelial metabolism (Arévalo Turrubiarte et al., 2016). The statistical analysis of MTT data evidenced that PPE at 0.1 μ g/mL, 1.0 μ g/mL, 10 μ g/mL for 24 h, 48 h and 7 days were not cytotoxic compared to the control. Only at high concentration (100 μ g/mL), cell death was observed (Figure S2). Subsequently, the antioxidant activity of PPE was evaluated on the bovine cell line by treatments at non-cytotoxic concentrations (0.1, 1.0, and 10 μ g/mL); after 48 h hydrogen peroxide (H₂O₂) was added to induce intracellular ROS production. Alternatively, cells were also treated with lipopolysaccharide (LPS) as indirect antioxidant agent. Experimental results evidenced that after 3 h, PPE at 1.0 μ g/mL and 10 μ g/mL showed a protective effect against ROS production induced by H₂O₂ with a decrease of 14.3% and 42.2%, respectively, compared to the control. At the same time, PPE at 10 μ g/mL produced a

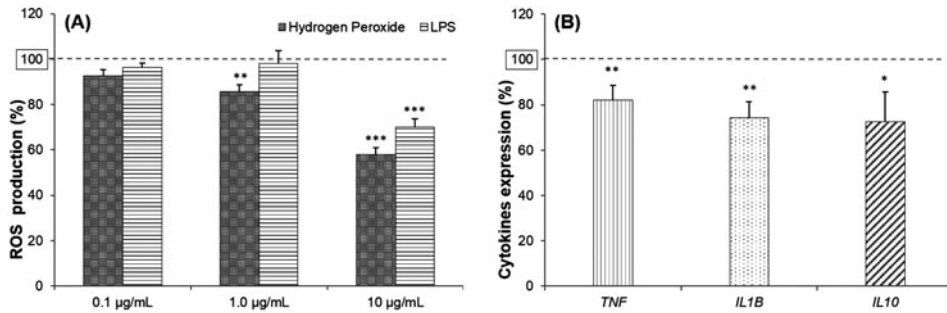


Figure 1. (A) ROS production after 48 h of treatment and 3 h of induction by H₂O₂ and LPS. Horizontal dashed line represents the control level (cells treated just with H₂O₂ or LPS). (B) Cytokines expression after 48 h of treatment with 10 µg/mL and 3 h of induction by LPS. Horizontal dashed line represents the control level (cells treated just with LPS). *P < 0.05; **P < 0.01; ***P < 0.001.

ROS decrease of 30% after the addition of LPS whereas at 1.0 µg/mL the extract resulted ineffective (Figure 1A). In both experiments, PPE at 0.1 µg/mL did not produce ROS decrease. The antioxidant effect of PPE at 10.0 µg/mL was confirmed by the reduction of malondialdehyde (MDA) compared to the control (31.1%, $p < 0.001$, data not shown). In fact, MDA is one of the products deriving from lipid peroxidation induced by oxidative processes and thus widely used as biomarker (Ayala et al., 2014). Punicalagin, the main component of PPE, could be responsible of ROS and MDA decreases as demonstrated by both *in vitro* and *in vivo* experiments on Caco-2 intestine cell line and rats, respectively (Omar et al., 2016; El-Missiry, et al., 2015). Finally, the anti-inflammatory effect of PPE at 10.0 µg/mL was evaluated on BME-UV1. Bovine cells were treated with PPE for 48 h, then LPS was added to induce inflammation and after 3 h, cytokines mRNA expressions: *TNF*, *IL1B*, and *IL10* were quantified. The experimental data evidenced that *TNF*, *IL1B*, and *IL10* were significantly different compared to the control (Figure 1B). In particular, *TNF*, *IL1B* and *IL10* decreased of 18.0, 25.7 and 27.5%, respectively. The trend of these results for the pro-inflammatory cytokines *TNF* and *IL1B* is in accordance to already reported in the literature about their expression in RAW 264.7 macrophage murine cells treated with the same concentration of PPE (Du et al., 2018). In addition, the reduced anti-inflammatory cytokine gene transcription *IL10* could be positively correlated with reduced transcription levels of pro-inflammatory cytokines during the inflammatory response (Dipasquale et al., 2018). Also the anti-inflammatory property of PPE could be related to punicalagin as demonstrated on rats and primary human epidermal keratinocytes (El-Missiry et al., 2015; Seok et al., 2018).

3. Conclusions

This study has evidenced for the first time that standardized pomegranate peel extracts, obtained from a by-products of fruits processing, reduced the oxidative stress and inflammatory status induced in bovine mammary epithelial cells BME-UV1. These results are promising for a possible use of these extracts as feed supplement for dairy cattle, in particular in the periparturient period. However, further studies need to

clarify the mechanisms of the observed effects and to investigate *in vivo* the fate of PPE across the gastrointestinal tract barrier after oral ingestion.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors gratefully acknowledge MIUR (Ministry for education, University and Research) for financial support (Law 232/216, Department of Excellence). The authors are also grateful to SIVAM Company (Casalpusterlengo, LO) which granted financial supported to the research.

ORCID

Umberto Bernabucci  <http://orcid.org/0000-0002-8126-3042>

Nicola Lacetera  <http://orcid.org/0000-0003-2088-2744>

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SUPPLEMENTARY MATERIAL

Antioxidant and anti-inflammatory effects of pomegranate peel extracts on bovine mammary epithelial cells BME-UV1

Fabio Mastrogiovanni,^a Roberta Bernini,^{a*} Loredana Basiricò,^a Umberto Bernabucci,^a Margherita Campo,^b Annalisa Romani,^b Luca Santi^a and Nicola Lacetera^a

^aDepartment of Agricultural and Forestry Sciences (DAFNE), University of Tuscia, Viterbo, Italy

^bDepartment of Statistics, Computing, Applications “Giuseppe Parenti” DiSIA, Phytolab, University of Florence, Florence, Italy

Contact

* Prof. Roberta Bernini, Department of Agricultural and Forestry Sciences, University of Tuscia, Via San Camillo De Lellis, 01100 Viterbo, Italy. E-mail: berninir@unitus.it
Phone: +39 0761357452. Fax: +39 0761357434

Experimental section

Chemicals

All chemicals including reference standards of the highest analytical grade, culture media, lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 were purchased from Sigma-Aldrich (Italy). PBS was furnished from Lonza (Swiss).

Plant material and preparation of PPE

Punica granatum L. fruits of the Wonderful variety were collected in Grosseto (Tuscany, Italy; Latitude:42°45'46"N; Longitude:11°06'33"E) as we previously reported (Imperatori et al., 2018). Details of the extraction are reported in a previous paper (Romani et al., 2012). Briefly, fresh pomegranate peels were frozen in liquid nitrogen, and grinded in a mortar. The powder was kept at 100°C with ultrapure water (MilliQ system waters Co., Milford, MA, USA) using a weight/volume=1/12. Finally, the extract was lyophilized using LYOVAC GT 2 and the liophiles were kept in a laboratory dryer in amber glass bottles at RT until use.

Chemical characterization

A sample of PPE was concentrated under vacuum (Rotavapor 144-R, Buchi, Switzerland) for HPLC/DAD/ESI-MS analysis. The chromatographic profile depicted in Figure S1 was recorded using a HP-1100 liquid chromatograph equipped with a DAD detector, a HP 1100 MSD API-electrospray (Agilent Technologies) detector, a Luna C18 column 250×4.60 mm, 5 µm (Phenomenex). The specific analytical conditions used for the HPLC analysis were as already reported (Romani et al., 2012). The ¹H-NMR spectrum depicted in Figure S1 was recorded using the 400 MHz nuclear magnetic resonance spectrometer Avance III (Bruker, Germany).

Cell lines

BME-UV1 cells respond to the epidermal growth factor and insulin-like growth factor I, associated to growth and development of mammary gland and show a morphology typical of luminal epithelial cells (Arévalo Turrubiarte et al., 2016). For these properties, they have been widely used as *in vitro* models to investigate milk production in dairy cows. BME-UV1 stabilized cell line was created from primary mammary epithelial cells transfected with plasmid that carries SV40 large T-antigen. The expression of SV40 establishes primary bovine mammary epithelial cells in culture

(Zavizion et al., 1996). BME-UV1 cell line (RRID:CVCL_W716) used in this study was kindly provided by Prof. Antonella Baldi (Department of Health, Animal Science and Food Safety, University of Milan, Italy). Cells were cultured in a medium composed by 50% DMEM-F12, 30% RPMI-1640, 20% NCTC-135, 10% fetal bovine serum, 0.1% lactalbumin hydrolysate, 1.2 Mm glutathione, 1 µg/mL of hydrocortisone, 0.5 µg/mL of progesterone, 10 µg/mL of L-ascorbic acid, and antibiotics (penicillin 100 IU/mL; streptomycin 100 µg/mL). Cells were kept at 37°C in humidified 5% CO₂ incubator (Basiricò et al., 2017); those used for the assays were between 39th and 43th passage.

Evaluation of the cytotoxic effect

Cell viability was measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay on BME-UV1. Different concentrations (0.1, 1.0, 10 and 100 µg/mL) of PPE were tested in order to evaluate the cytotoxic effects after 24 h, 48 h and 7 days. Cells were plated into 96-well microplates (Sarstedt AG & Co.) at 5×10^4 cells/well for the 24 h treatment, 2.5×10^4 cells/well for the 48 h and 2×10^3 cells/well for the 7 days. Plates were kept in incubator for 24 h to allow cells to adhere to the bottom. After cells adhesion, standard culture medium was replaced by 200 µL of each PPE dilutions or regular medium (as control). At the end of the exposure times treatments and control were removed and replaced with 100 µL of solution composed by regular medium and MTT to a final concentration of 0.5 mg/mL. After 3 h and 30 min of incubation supernatants were discarded and 100 µL of dimethyl sulfoxide (DMSO) were added to each well; plates were additionally incubated for 45 min and subsequently read at 595 nm by microplates reader (Tecan SunriseTM), absorbance data were recorded.

Evaluation of the antioxidant effect

To evaluate the possible protective effect against oxidative stress, Reactive Oxygen Species (ROS) and malondialdehyde (MDA) productions were measured after induction with hydrogen peroxide (H₂O₂) and lipopolysaccharide (LPS).

ROS evaluation

ROS production was evaluated after 48 h of treatment; 2.5×10^4 cells/well were plated in 96-well black microplate (Lumox®, Sarstedt AG & Co.) for fluorescence reading. After 24 h from plating, cells were treated with different concentrations of PPE: 0.1, 1.0

and 10 µg/mL and with regular medium as control. After 48 h, H₂O₂ was added as direct oxidant agent (final concentration of 50 µM) or LPS from *Escherichia coli* 055:B5 as indirect oxidant agent (final concentration of 20 µM) to both the treated cells and control; subsequently, the microplates were incubated for an additional 3 h. Finally, the supernatants were discarded, and cells were washed twice with PBS and then incubated with 100 µL of 2',7'-dichlorodihydrofluorescein diacetate 20 µM in PBS at 37°C; 40 min later the plates were read using a microplate reader (Ultimode Detector DTX 880, Beckman Coulter Inc.) at 485 nm (wavelength of excitation) and 535 (wavelength of emission).

MDA evaluation

MDA production was evaluated after 48 h of treatment (Ayala et al. 2014); 2 x 10⁶ cells were plated in 75-cm² tissue culture flasks (Costar, Corning, NY) using the previously described culture medium. After 24 h the medium was replaced by 10 µg/mL of PPE or with fresh medium (control) for 48 h. Then, H₂O₂ was added (50 µM of final concentration) and after 3 h, the cells, detached with a solution of trypsin/EDTA, were centrifuged at 4.500 × g for 5 min at 4°C. The supernatant was discarded and the pellet was dissolved in 200 µL of PBS; subsequently, by 2 cycles of sonication at 100 W for 30 s cells were lysed, centrifuged at 15.000 × g for 5 min at 4°C, and stored at -80°C until analysis. MDA concentrations were determined using the proper detector kit (Abcam, Cambridge, UK). Absorbance was measured at 540 nm by microplate reader (Tecan Sunrise™).

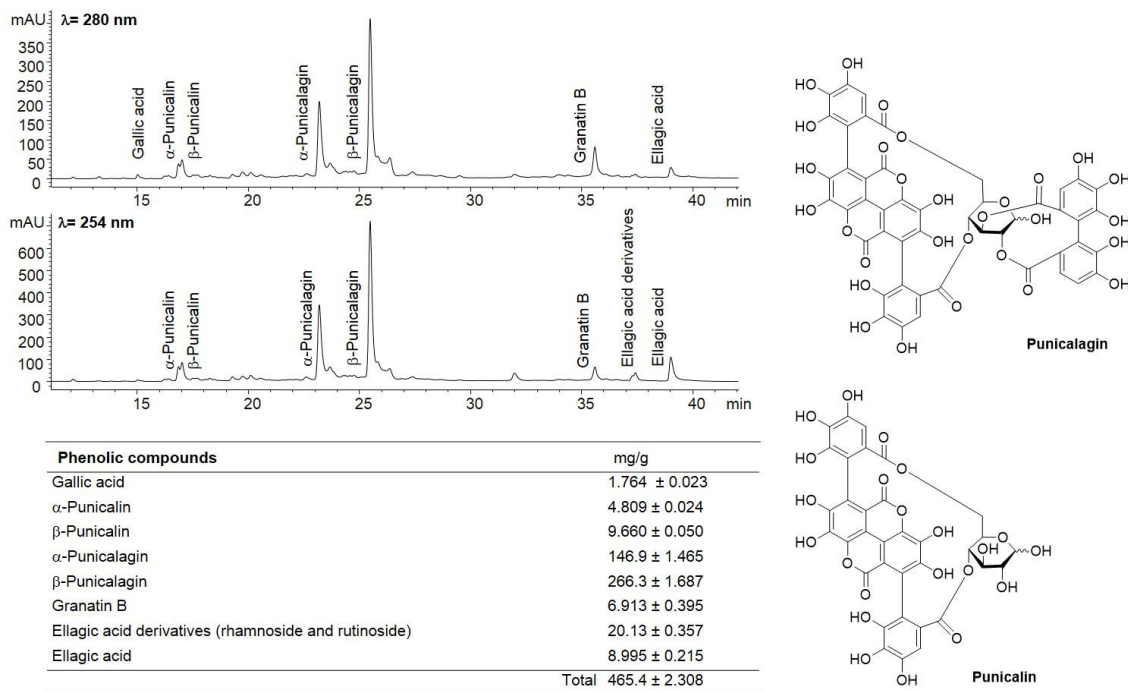
Evaluation of pro- and anti-inflammatory interleukins expressions

The effect of PPE as pro- or anti-inflammatory was evaluated through mRNA expression after 48 h of incubation with 10 µg/mL of treatment and induction of inflammation by LPS. 2 x 10⁶ cells were plated in 75-cm² tissue culture flasks; after 24 h, the medium was discarded and replaced by fresh medium containing 10 µg/mL of PPE or with fresh medium as control for 48 h. Then, LPS (at final concentration of 20 µM) was added and incubated for 3 h. Cells were detached using a solution of trypsin/EDTA and centrifuged at 130 × g for 5 min at 37°C. Finally, the supernatant was discarded to recover the pellet. After the addition of 1 mL of PBS, the suspension was centrifuged at 130 x g for 5 min, and after supernatant removal, 1 mL of Qiazol was added to each tube and stored at -80°C until use. The RNA quantification was made using Quant-iT RNA assay Kit (Invitrogen, Carlsbad, CA, USA) and fluorescence was

measured at 644 nm (wavelength of excitation) and 673 (wavelength of emission). Reverse transcription was performed using 1 µg of total RNA and employing Quantitect reverse transcription kit (Qiagen) in a volume of 20 µl on a PCR Express thermal cycler (Hybaid, Ashford, UK). qPCR was performed in the LightCycler® 2.0 (Roche; Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's recommendations for probes. For each sample was also quantified an housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase) to normalize the cytokines expression data by $\Delta\Delta\text{Ct}$ method. More information about primers used, can be find in Table S1.

Statistical analysis

All data were compared to the control, converted in percentages and analyzed by ANOVA using Statistica 10 Software package (Stat Soft, Inc., USA). Data were presented as LS means and standard error of the means. The significance of the comparison was assessed by the Fisher's Least Square Difference (LSD) test. Significance was attributed at $P < 0.05$.



All determinations were carried out in triplicate; results were expressed as mean values, the standard errors were always <5%.

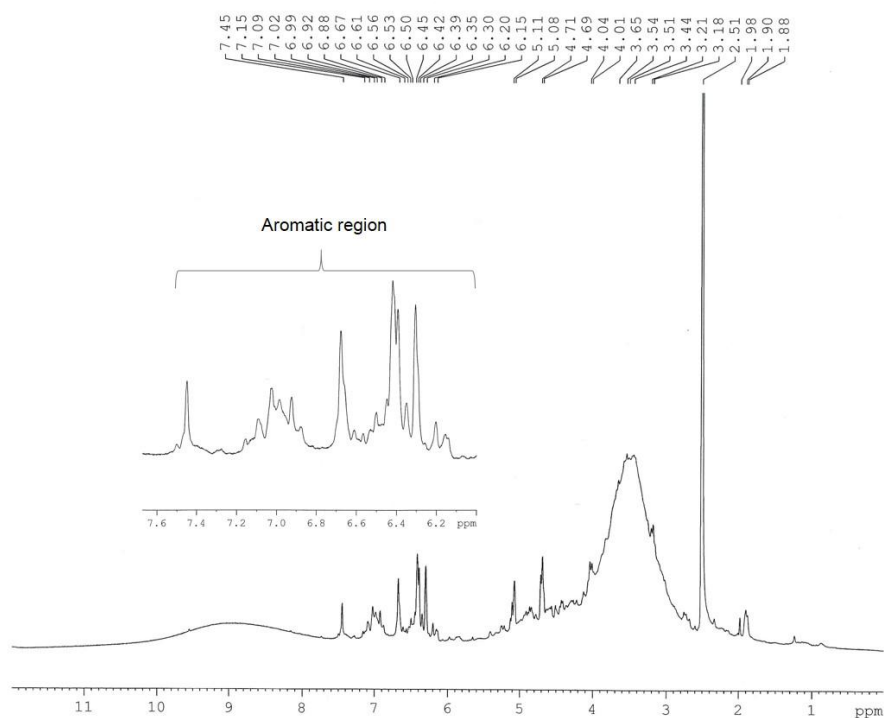


Figure S1. Chemical structures of the hydrolyzable tannins found into PPE; HPLC profile and ^1H NMR spectrum of PPE (Imperatori et al., 2018).

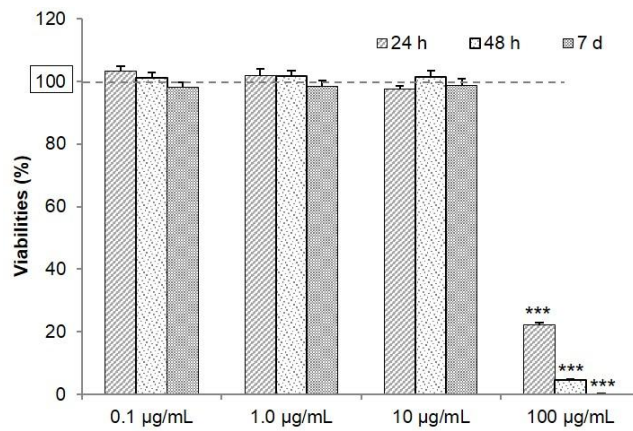


Figure S2. Cell viability after 24 h, 48 h and 7 days of treatment. Horizontal dashed line represents the control level, namely cells non-treated with the extract. All treatments were compared to the respective control. *** $P < 0.001$.

Table S1. DNA sequences of bovine sense and antisense primers and probes used for real-time PCR-analysis Gene Primers and probes Temperature of annealing ($^{\circ}\text{C}$).

Gene	Primers and probes	T of annealing ($^{\circ}\text{C}$)
<i>TNF F</i>	TCTTCTCAAGCCTCAAGTAACAAGT	60
<i>TNF R</i>	CCATGAGGGCATTGGCATACT	
<i>TNF P</i>	FAM-AGCCCACGTTGTAGCCGACATCAACTCC-TAMRA	58
<i>IL1B F</i>	TCCACCTCCTCTCACAGGAAA	
<i>IL1B R</i>	CTCTCCTTGCACAAAGCTCATG	
<i>IL1B P</i>	FAM-CACCACTTCTCGGTTC-MGB	60
<i>IL10 F</i>	CTTGTCGGAAATGATCCAGTTTT	
<i>IL10 R</i>	TTCACGTGCTCCTTGATGTCA	
<i>IL10 P</i>	FAM-CCACAGGCTGAGAACCACGGGC-TAMRA	60
<i>GAPDH F</i>	GCATCGTGGAGGAGGGACTTATGA	
<i>GAPDH R</i>	GGGCCATCCACAGTCTTCTG	
<i>GAPDH P</i>	FAM-CACTGTCCACGCCATCACTGCCA-TAMRA	

TNF=tumor necrosis factor- α ; *IL1B*=interleukin-1 β ; *IL10*=interleukin-10; *GAPDH*=glyceraldehyde 3-phosphate dehydrogenase; *F*=Forward; *R*=reverse; *P*=probe.

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