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Punicalagin isolated from *Punica granatum* husk can decrease the inflammatory response in RAW 264.7 macrophages

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ABSTRACT

Punicalagin, a natural occuring phytochemical extracted from pomegranate *(Punica granatum*) husk shows antioxidant and anti-tumoral activities. Previous studies have shown that *Punica granatum* can scavenge reactive oxygen intermediates (ROIs) and suppress the biosynthesis of prostaglandins (PGs). However, the anti-inflammatory property of punicalagin has not yet been elucidated. For this aim, we purposed to display the anti-inflammatory effects of punicalagin via measuring prostaglandin E_2 (PGE_2) and nitric oxide (NO) production and cyclooxygenase-2 (*COX-2*) and inducible nitric oxide synthase (*iNOS*) gene expression in LPS induced RAW 264.7 macrophages. LPS-stimulated RAW 264.7 macrophages were treated with punicalagin at concentrations of 0-10 μM. NO levels in all groups were measured by the Griess method and PGE_2 levels by ELISA kit. *COX-2* and *iNOS* gene expression levels were measured using Real Time PCR. According to our experiments, punicalagin decreased the production of PGE_2 and NO by RAW 264.7 macrophages in a dose-dependent manner without affecting the viability of cells. Punicalagin attenuated the mRNA expression of *iNOS* and *COX-2* of murine macrophages in a concentration dependent manner. Thus, the inhibition of NO and PGE_2 production is at least partly because of the suppression of the transcription of the iNOS and COX-2 gene, respectively. In conclusion, punicalagin is a potent natural compound in inhibiting the inflammatory mediators. Its action can be delivered *in vivo* through an appropriate feeding scheme. Because the lower toxicity of punicalagin, it might be a suitable compound that can be used for clinical applications.

Key Words: Punicalagin, inflammation, RAW 264.7 macrophages, nitric oxide, prostaglandin E_2

Introduction

Inflammation is an important immune response to defend the body from invasion of tissue injury, chemical irritation or microbial pathogen infection. It has a vital function in the immune response to combat foreign invaders of the body (1,2). Under inflammatory condition, mast cells secrete chemokines and pro-inflammatory cytokines that cause angiectasis, increase permeability, and subsequently recruit leukocytes, macrophages and a wide range of immune cells to invade the affected area. Leukocytes and macrophages infiltrate the damaged regions, remove the irritation and repair the tissue. RAW 264.7 murine macrophages obtained from the tissue of Abelson murine leukemia virus-induced tumor in male adult BALB/c Mus musculus is one of the common cell line model in inflammatory studies since its lipopolysaccharide (LPS) induced state exhibits these typical functional and morphological phenotypes (3,4). LPS triggers the secretion of pro-inflammatory mediators such as prostaglandin E_2 (PGE_2) and nitric oxide (NO) which are created by the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Inhibition of these pro-inflammatory mediators is a critical goal to protect the organism against the inflammation. Thus, many compounds believed to have anti-inflammatory activity have been applied to RAW 264.7 macrophages for treating the inflammatory response (5,6).

Pomegranate (*Punica granatum* L., *Punicaceae*) has been widely used in ayuverdic medicine and other traditional medicine. Pomegranate leaf extract exhibits antihelmintic, antiparasitic, antidiarrhoeal, antioxidant, antitumoral and anti-inflammatory properties (7). Two types of polyphenols are found in pomegranate; anthocyanins (delphinidin, cyaniding and pelargonidin), responsible for the red color of the fruit, and hydrolysable tannins, mainly ellagitannins. These polyphenols are responsible for 90% of the antioxidant capacity of pomegranate and punicalagin alone undertakes more than 50% of this antioxidant property (8). Punicalagin is a hydrolyzable tannin and it is

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considered to be the main compound in pomegranate husk (9). Punicalagin is reported to have anti-cancer, antiatherosclerotic and anti-obesity properties (10). Although many researches have reported the anti-inflammatory feature of pomegranate leaf extract, effects of punicalagin obtained from pomegranate leaf extract is not known clearly (11,12).

Better discerning of the anti-inflammatory effects of punicalagin may lead to further development of new strategies for dysregulated inflammatory prevention at many sites. Therefore, in this study, we purposed to investigate the anti-inflammatory effects of punicalagin via measuring PGE_2 and NO production and *COX-2* and *iNOS* gene expression in LPS induced macrophages.

Materials and methods

Cell culture

RAW 264.7 macrophages were purchased from American Type Culture Collection (ATCC) and the cells were incubated at 37°C and 5% CO_2. All the chemicals used in our study were purchased from Sigma-Aldrich and Merck companies and all products were in cell culture purity. The cells were passaged when they were cultivated at a density of 70~80 % full of the dish. Medium was removed and 1× Phosphate buffered saline (PBS) was used to wash the dish. We subsequently tapped the dish and used fresh medium to wash down the cells. The fallen cells were collected in centrifuge tube, and we centrifuged the tube with 1000 rpm for 5 minute. After that, the supernatant was sucked up and the pellet was dispersed by fresh medium. Finally, the cells were seeded in dishes or wells with a proper density.

After the cells grew stably and reached at a density of $2\text{-}3\times10^6$ cells/mL, the medium was removed and 1× PBS was used to gently wash the dish. The medium was replaced serum-free DMEM. The cells were induced by incubation in medium containing 0.1 μg/mL LPS (E. coli 0127: E8). Punicalagin was dissolved in DMEM. These dissolution compounds were treated cells together with LPS.

Cell viability assay

The cellular toxicity of punicalagin was determined by (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (13). After 24 hr of incubation with the cells, punicalagin were removed by washing the cultured cells three times with medium. Then, the cells were collected by centrifugation and the pellets were suspended in fresh medium. The MTT solution was prepared using phosphate buffered solution (PBS) at 5 mg/mL concentration. MTT solution was added to the culture plate with cells and then incubated at 37°C for 4 hours. The solution of 0.4 N HCl in isopropyl alcohol was added to the culture well to dissolve the formed tetrazolium salt. The results were measured by ELISA which read the absorption at 540 nm in ELISA microplate reader (Thermo Fisher Scientific, Wathham, MA, USA). The experiment was repeated by three times. Medium without cells was used as blank.

Determination of NO production

Nitrite and peroxynitrite levels in the cell supernatant were measured using the Griess method (14) to determine NO level in the cell culture medium. Nitrite and peroxynitrite are stable end products of NO in *in vitro* system. By this way, the level of NO synthesis by iNOS can be evaluated. Griess reagent was prepared by mixing 1 volume of 1% sulfanilamide solution and 1 volume of 0.1% N-l-naphthyl ethlenediamine dihydrochloride prepared in 2.5% H_3PO_4. Tested cells were treated by 0.1 μg/mL LPS. The tested compounds were added in gradient concentrations into the medium with cultured cells before the adding of the stimulants. After 24 hr, 48 hr and 72 hr of stimulation, 50 μL of supernatant of the culture wells was taken and mixed with 50 μL freshly prepared Griess reagent. After incubation at room temperature for 10 minutes, the ELISA plate was centrifuged for 5 minutes at 1000 rpm. The results were obtained from ELISA by reading the absorption at 540 nm. This reaction was repeated three times for each sample. Accumulation of nitrite was calculated as nmol of nitrite per 10^6 cells from three independent experiments. The culture medium without cells was used as blank. Sodium nitrate ($NaNO_2$) was diluted to 3 μM, 25 μM, 50 μM and 100 μM and used as standard solutions.

Determination of PGE_2 production

Prostaglandins (PGs) are lipid compounds derived from arachidonic acid. Like all PGs, PGE_2 can be produced by several tissues including the gut, the uterus, blood vessels, bladder, placenta, brain and cells of the immune system. In some pathologic conditions such as tissue injury, inflammation and many cancers the production of PGE_2 is increased in bodily fluids. The principle of PGE_2 immunoassay is that PGE_2 from culture medium competes with horseradish peroxidase (HRP)-labeled PGE_2. After both PGE_2 and HRP-labeled PGE_2 bind to the antibody sites, a substrate

solution is added to evaluate the bound enzyme activity. The concentration of PGE_2 present in the sample is inversely proportional to the intensity of the color at the end of the immunoassay.

Gene expression analysis

RNA extraction: Culture medium was removed after 4 or 5 hours of incubation. 300 μL lysis buffer was added per 10^6 cells. Then, 100 μL of protein/DNA precipitating buffer was mixed and centrifuged to remove the protein and DNA. Supernatant was removed to new eppendorf tube and mixed with 300 μL isopropanol. The tube was put in -70 °C freezer for more than 24 hours to precipitate RNA. RNA pellet was washed by 70% ethanol and then dissolved in DEPC-water. For tissue samples, TriReagent (Sigma) was used to homogenize the tissue samples in homogenizers. Chloroform was added to remove DNA and protein. After centrifugation, the water phase was removed to a new tube and 1 volume isopropanol was mixed to precipitate RNA. RNA was washed by 70% ethanol to remove isopropanol and dissolved into DEPC-water. The concentration of extracted RNA was quantitated by measuring the absorption in OD_{260} and OD_{280} in spectrophotometer. The ratio of OD_{260}/OD_{280} is between 1.6 to 1.8 for samples were used for the following experiments.

cDNA synthesis: Total RNA extracted from cultured cells and tissue samples were used as template for reverse transcription. 300 ng total RNA was added to 25 μL total volume of reaction mix containing 0.6 mM dNTPs (Promega), 10 units Rnase inhibitor (Clonetech), O1igo$(dT)_{15}$ primer (Promega) and 100 units of MMLV-RT (Promega). The whole reaction mix was incubated for 20 minutes at 42°C. Then, the activity of reverse transcriptase was stopped via heating at 94°C for 5 minutes. The synthesized cDNA was chilled on ice immediately for PCR reaction or stored at -70°C for later analysis.

Real time PCR Analysis: The production of PCR products was determined by measuring the SYBR Green fluorescence signal. SYBR Green DNA enters small, large cavities in double strands. The unbound dye in the solution emits very low fluorescence. However, as the binding of SYBR Green to DNA increases, the fluorescence emission at a wavelength of 530 nm rises. During PCR, the increase in SYBR Green fluorescence composition increases with the proportion of directly produced double-stranded DNA. Real Time PCR was performed in the direction of the manufacturer using the Invitrogen Universal EXPRESS SYBR Greener qPCR SuperMixes and Two-Step qRT-PCR kit on the Roche LightCycler 480 II device. The following PCR primers were used; (*β-actin* forward; 5'-AGG TCA TCA CTA TTG GCA AC-3', *β-actin* reverse; 5'-ACT CAT CGT ACT CCT GCT TG-3', *COX-2* forward; 5'-GTC TGA TGA TGT ATG CCA CAA TCT G-3', *COX-2* reverse; 5'-GAT GCC AGT GAT AGA GGG TGT TAA A-3', *iNOS* forward; 5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3' and *iNOS* reverse, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3' (15). In our study, β-actin gene was used as the house-keeping gene. Concentration values of *iNOS* and *COX-2* gene expression of each sample were proportional to the concentration of β-actin of the same sample. The obtained values were multiplied by 100 and gene expression levels were calculated for each sample and the results obtained were compared. Relative quantitation was also used when expression changes were calculated and the expression of *iNOS* and *COX-2* genes in LPS and different concentration of punicalagin treated RAW 264.7 macrophages was expressed as folds, assuming gene expression values 1 in no-treatment controls.

Statistical analysis

All experiments were repeated three times. The values were determined as mean ± SE (standard error). SPSS (version 15.0 for windows) was used for determining the treatment effects by one-way ANOVA. The difference or correlation was considered to be statistically significant if $p < 0.05$ in the obtained results.

Results

Cytotoxicity of punicalagin in RAW 264.7 macrophages

Prior to evaluation of the NO and PGE_2 inhibitory effect of punicalagin, we first determined its non-cytotoxic concentration in LPS-stimulated RAW 264.7 macrophages using MTT assays. In this study we have applied to punicalagin RAW 264.7 macrophages at concentrations of 0, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 100, 150, 150, 250 and 500 μM. Concentrations of 0, 2.5, 5, 7.5 and 10 μM of punicalagin were used in our experimental study, with a maximum of 10% cytotoxicity, in other words at least 90% cell viability (Figure 1). In our study, the LD50 concentration of punicalagin for the RAW 264.7 macrophages was found to be 895.01 μM.

Effects of punicalagin on NO and PGE_2 production in LPS-stimulated RAW 264.7 macrophages

We measured NO and PGE_2 production in LPS-stimulated RAW 264.7 macrophages for determining the possible anti-inflammatory effects of punicalagin. As shown in Figure 2, NO production and in Figure 3, PGE_2 production was substantially higher in LPS-treated cells than in the untreated control cells ($p<0.05$). The addition of 2.5, 5, 7.5 and 10 µM punicalagin caused 31.74%, 38.02%, 43.35% and 50.18% reduction in LPS-induced NO production, respectively (Figure 2 and 3) and the addition of 2.5, 5, 7.5 and 10 µM punicalagin caused 5.73%, 12.54%, 21.09% and 25.34% reduction in LPS-induced PGE_2 production, respectively (Figure 3). The decrease in NO production was statistically significant for all punicalagin concentrations ($p<0.05$). The decrease in PGE_2 production was not statistically significant in the groups treated with punicalagin at concentrations of 2.5 and 5 µM ($p>0.05$), but a statistically significant difference was observed in the groups treated with punicalagin at concentrations of 7.5 and 10 µM ($p<0.05$).

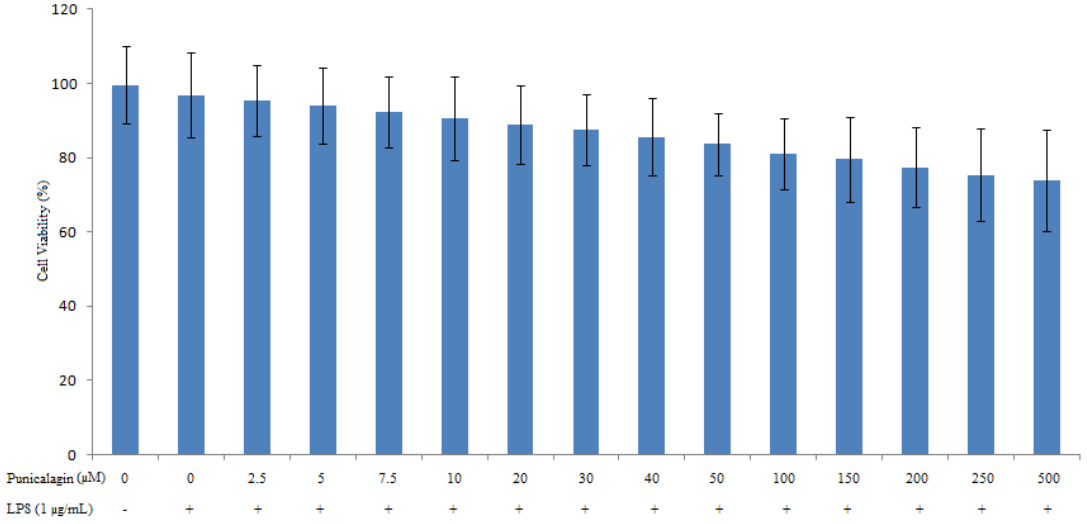
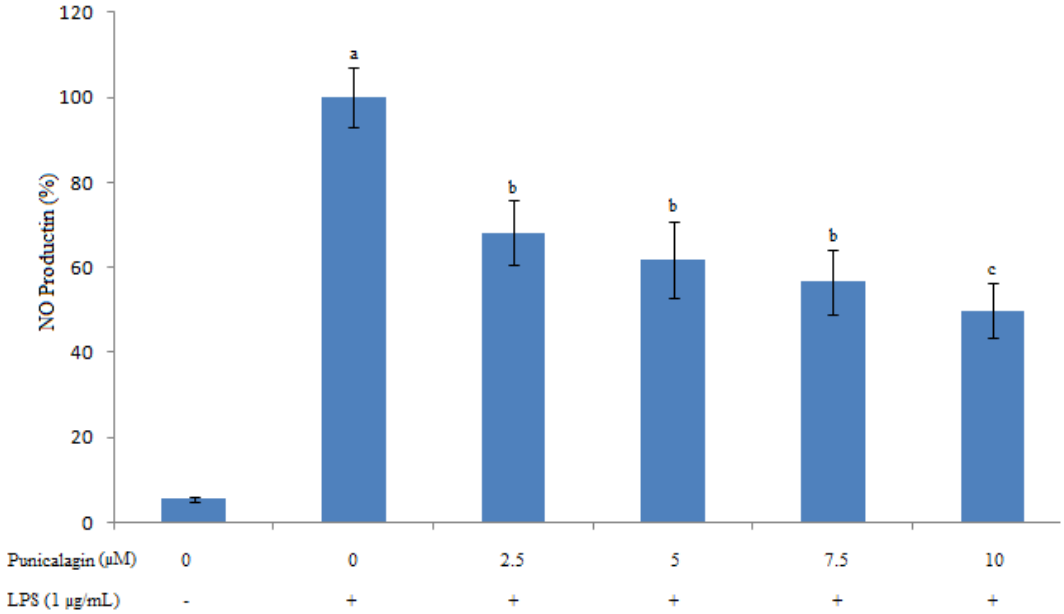


Fig. 1. Effect of punicalagin on the cell viability of RAW 264.7 macrophages. There was no statistically differences between the groups ($p>0.05$).



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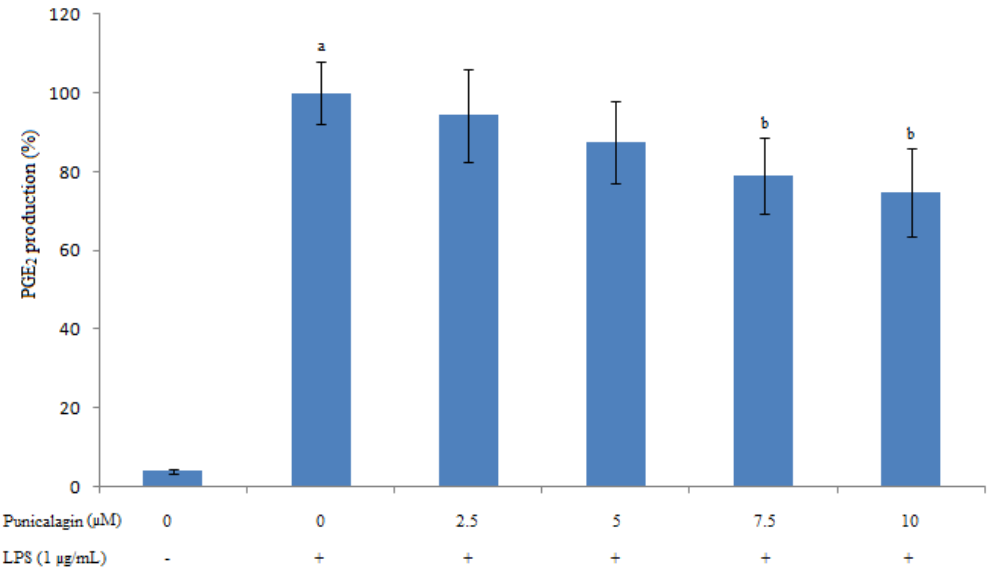

Fig. 3. Effects of punicalagin on LPS-induced PGE_2 production in RAW 264.7 macrophages. [a] $p<0.05$ compared with the control cells, and [b] $p<0.05$ compared with the LPS-only treated cells.

Effect of punicalagin on iNOS and COX-2 mRNA expression in LPS-stimulated RAW 264.7 macrophages

RT-PCR was performed to determine whether the inhibitory effects of punicalagin on NO and PGE_2 production were related to the mRNA expression of *iNOS* and *COX-2*. As shown in Figure 4, *iNOS* mRNA expression level and in Figure 5, *COX-2* mRNA expression level were substantially higher in LPS-treated cells than in the untreated control cells ($p<0.05$). The addition of 2.5, 5, 7.5 and 10 µM punicalagin caused 34.51%, 46.09%, 54.18% and 62.7% reduction in LPS-induced *iNOS* mRNA expression, respectively (Figure 4) and the addition of 2.5, 5, 7.5 and 10 µM punicalagin caused 32.01%, 40.84%, 48.65% and 55.68 % reduction in LPS-induced *COX-2* mRNA expression level, respectively (Figure 5). It has been demonstrated that all of the punicalagin concentrations performed resulted in a statistically significant decrease in both *iNOS* and *COX-2* gene expression ($p<0.05$).

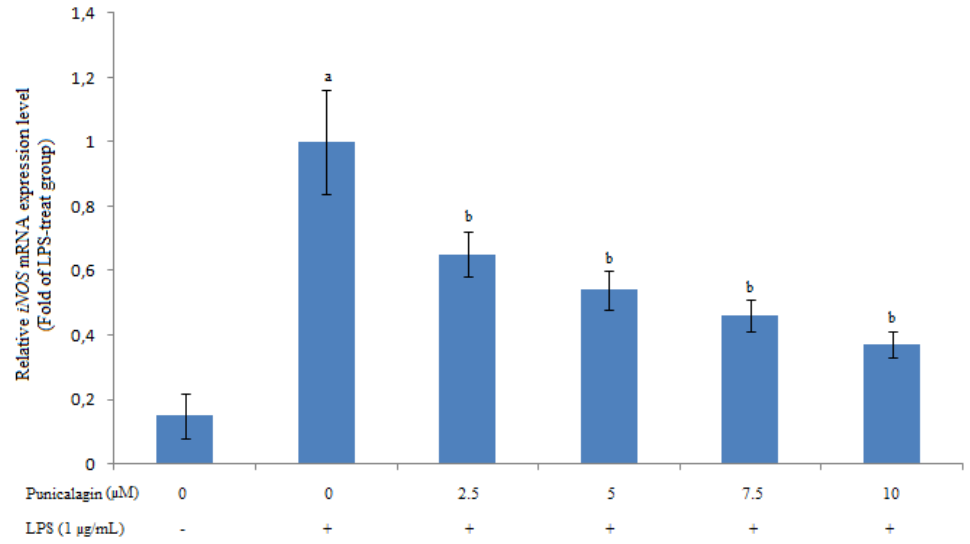

Fig. 4. Effects of punicalagin on LPS-induced *iNOS* gene expression in RAW 264.7 macrophages.
[a] $p<0.05$ compared with the control cells, and [b] $p<0.05$ compared with the LPS-only treated cells.

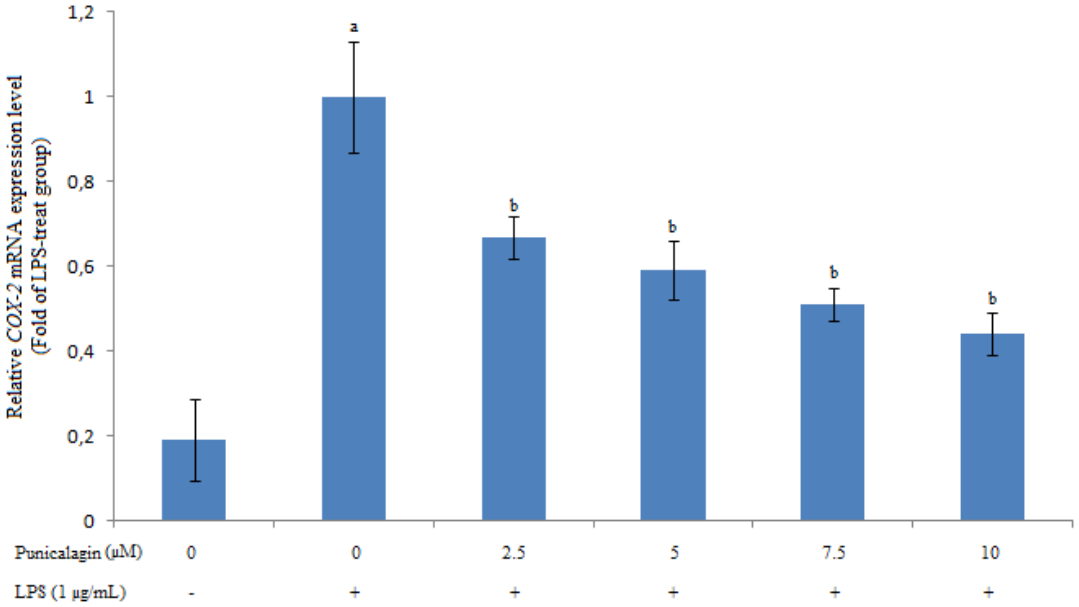

Fig. 5. Effects of punicalagin on LPS-induced *COX-2* gene expression in RAW 264.7 macrophages.
[a] $p<0.05$ compared with the control cells, and [b] $p<0.05$ compared with the LPS-only treated cells.

Discussion

Inflammation is a protective reaction orchestrated by the immune system to noxious stimuli and events, such as infection, tissue injury, and tissue malfunction. Its main purpose is to facilitate the removal of the stimuli, as well as the initiation of the healing process for the damaged cells or tissues. Inflammation is categorized as acute, localized chronic, and systemic chronic (16). The cellular and molecular mechanisms involved in acute inflammation, which are triggered commonly by infection and to some degree tissue injury, are well understood and characterized. However, much less is known about the causes and mechanisms of localized and systemic chronic inflammation, which are associated with a plethora of diseases - diabetes, obesity, insulin resistance, coronary heart disease, cardiovascular disease, cancer, Alzheimer's, asthma, inflammatory bowel disease, and arthritis (17).

Since murine macrophages, RAW 264.7 cell lines, are one of the common cell line models in inflammatory studies, we applied them as a model cell line in our study. They were isolated from the tissue of Abelson murine leukemia virus-induced tumor in male adult BALB/c mice. The culture property of the cells is adherent. In the research, a classical model of macrophage stimulation is bacterial endotoxin, lipopolysaccharide (LPS) (18,19). It is a classical and well-known model of macrophage stimulation. After LPS binds to the receptor, the signaling pathways are induced involved in modulating activation Nuclear Factor-κB (NF-κB). The activation of NF-κB has been demonstrated as a crucial role to regulate gene expressions in LPS-stimulated inflammatory responses, including NO and PGE_2 (19).

Many plant extracts can be beneficial for ameliorating LPS-induced inflammation in folk medicine. For this purpose, many fruits as well as pomegranate have been widely used for their anti-inflammatory properties (20). Many have reported the anti-inflammatory function of pomegranate in both *in vitro* and *in vivo* studies (21,22). Pomegranate juice has been shown to promote antioxidant and antiinflammatory effects by inhibiting oxidative destruction of NO. *In vitro* experiments have shown that pomegranate seed oil obtained by cold pressing suppresses cyclooxygenase and lipoxygenase enzymes (12). In another *in vitro* study, pomegranate extract has been shown to significantly suppress the subset of matrimal metalloproteinases of collagenase enzymes, which play a role in the construction and degradation of high resolution and extracellular matrix of the osteoarthritic joint (23). Treatment of pomegranate extract suppresses the degradation of proteoglycan stimulated by interleukin-1 in human femoral osteoarthritic chondrocytes and

synthesis of cellular matrix metalloproteinases. In osteoarthritic chondrocyte cultures it has been shown that pomegranate extract inhibits collagen degradation, and may possibly suppress joint destruction in patients with osteoarthritis (24). However, the anti-inflammatory properties of pomegranate have not yet been demonstrated in clinical trials (12). Although these studies as well as other studies not mentioned here suggest that pomegranate extract hold good potential as anti-inflammatory agent, data on the effects of punicalagin isolated from pomegranate husk is lacking (23,24). The current study has been performed to enlighten the anti-inflammatory properties of punicalagin on LPS-induced RAW 264.7 macrophages.

Since the anti-inflammatory activity of punicalagin is independent of its cytotoxicity, we aimed primarily to determine the subcytotoxic concentrations of punicalagin in this study. For this reason, cell viability ratios were examined by MTT assay of RAW 264.7 macrophages incubated with different concentrations of punicalagin for 24 and 48 hours. The study showed that there was no significant change in viability of cells treated with 2.5, 5, 7.5 and 10 μM punicalagin, and punicalagin at concentrations of 20 μM and higher caused significant cytotoxicity in RAW 264.7 macrophages. For this reason, in our study we used 0-10 μM punicalagin concentrations having no cytotoxicity to RAW 264.7 macrophages.

Our study has shown that all applied concentration of punicalagin decreased LPS-induced NO production and gene expression of *iNOS* and *COX-2* in RAW 264.7 macrophages, significantly. Similarly, all applied concentration of punicalagin decreased LPS-induced PGE_2 production but only 7.5 and 10 μM punicalagin decreased PGE_2 production significantly. This indicates that punicalagin inhibited LPS-induced NO and PGE_2 production and *iNOS* and *COX-2* gene expression in RAW 264.7 macrophages in a dose-dependent manner and the decline in NO and PGE_2 production by punicalagin is a result of the inhibition of *iNOS* and *COX-2* gene expression.

In conclusion, it has been revealed that punicalagin isolated from *Punica granatum* inhibits inflammation via suppressing *iNOS* and *COX-2* gene expression and NO and PGE_2 production. The data shows that punicalagin holds great potential against the inflammatory pathway. This study has led to the conclusion that regular consumption of *Punica granatum* may help reducing the inflammatory response.

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