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PAPER

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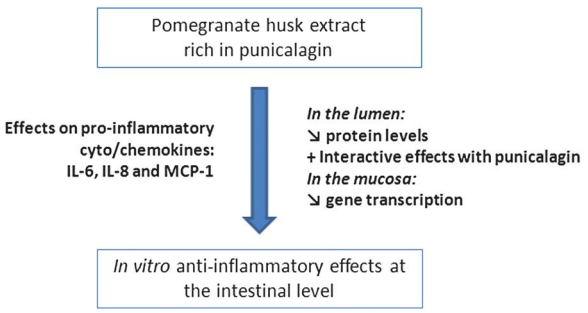
Pomegranate husk ellagitannins decrease intestinal inflammation by acting both on the gene transcription and protein levels of pro-inflammatory cyto/chemokines, the later phenomenon being possibly due to direct molecular trapping.



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PAPER

Anti-inflammatory effects of pomegranate (*Punica granatum* L.) husk ellagitannins in Caco-2 cells, an *in vitro* model of human intestine

Sylvie Hollebeeck,[a] Julie Winand,[a] Marie-France Hérent,[b] Alexandrine During,[a] Joëlle Leclercq,[b] Yvan Larondelle[a] and Yves-Jacques Schneider*[a]

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This study aimed at evaluating the anti-inflammatory properties of a pomegranate fruit husk (PomH) polyphenolic extract, rich in punicalagin, using Caco-2 cells, an *in vitro* model of human intestinal epithelium. Differentiated cells in bicameral inserts were pretreated or not with a PomH extract or punicalagin, as reference, at the apical side, representing the intestinal lumen. Inflammation was then induced with a cocktail of cytokines (Il-1β, TNFα and IFNγ) and LPS. After 24 h incubation, 3 pro-inflammatory markers, *i.e.*, interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1, were assayed both at their gene transcription (qRT-PCR) and secretion (ELISA) levels. As previously described, the pro-inflammatory cocktail significantly stimulated these 3 markers, at the gene transcript and secretion levels. In inflamed cells, a significant down-regulation of the transcription of the genes encoding IL-6 and MCP-1 was observed in the presence of the PomH extract or punicalagin, while IL-8 transcription was unaffected. Both treatments also decreased the amounts of the 3 proteins with dose-response effects, but only in the apical compartment. A lowered ELISA response was also observed when either IL-6, IL-8 or MCP-1 were mixed with punicalagin in a cell-free culture medium, indicating a direct molecular interaction. In conclusion, the punicalagin-rich PomH extract tested showed anti-inflammatory properties in the Caco-2 *in vitro* intestinal model. It acted both on the pro-inflammatory gene transcription and protein levels, the later phenomenon being possibly due to a direct molecular trapping. These data suggest that pomegranate husk could be an interesting natural source contributing to prevent intestinal chronic inflammation.

Introduction

Inflammatory bowel diseases (IBDs), *e.g.* Crohn's disease and ulcerative colitis, are a group of chronic inflammatory disorders of the gastrointestinal tract characterized by a relapsing and remitting course. A normal mucosa is characterized by a well controlled balance of the intestinal immune system, a "physiological" inflammation, whereas a pathological inflammation is the product of a dysregulation of this balance.

The inflammatory response is coordinated by a complex network of mediators released from epithelial cells and from cells within the underlying *lamina propria* (*e.g.*, leukocytes, neurones, dendritic cells). These cells exchange regulatory signals *via* the production of mediators (cytokines, chemokines, growth factors, adhesion molecules and neuropeptides) and/or cell events (proliferation and apoptosis).[1] Intestinal epithelial cells can be therefore considered as integral components of the immune system by taking actively part to both innate and acquired immune responses through the secretion of a variety of inflammatory mediators.[2]

Cytokines are secreted mainly by immune cells, but also, among others, by the intestinal epithelial cells. They play a key role in the regulation of the mucosal immune system upon binding to specific cellular receptors. They are also key actors in the disruption of the 'physiological' intestinal inflammation.[3] Cytokines facilitate the intercellular communication, stimulate the proliferation of antigen specific effector cells and mediate the local and systemic inflammation by auto-, *para*- and endocrine signaling.[4] Three types of cytokine functions may be identified in mediating the inflammatory response: (i) induction of inflammation by stimulating the proliferation of T cells, promoting leukocyte infiltration, and facilitating intercellular signaling (interleukin (IL)-6 and tumor necrosis factor (TNF)-α); (ii) limitation of the expansion of leukocytes and restoration of the activated immune cells and macrophages to their resting states (IL-10); and (iii) modulation of the action of pro-inflammatory cytokines (*e.g.*, interferon (IFN)-γ and IL-18).[4] Chemokines are a family of small cytokines such as IL-8 and monocyte

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chemoattractant protein (MCP)-1 that play a role in leukocyte attraction from the circulatory system to local inflammatory sites.[5]

Cytokine and chemokine networks play an important role in the initiation and perpetuation of the inflammatory reaction in IBD. Effective immune-based therapies have already been developed, such as those based on monoclonal antibodies to the cytokines IL-6[6] and TNF-α (Infliximab, CDP571), and recombinant Interleukin-10 (Tenovil).[4] In the same way, the development of selective inhibitors for chemokines or chemokine receptors are of great interest as potential therapeutic strategies in IBD.[7] Beside therapeutic approaches, there is however, a particular interest to develop natural prevention measures because of the side effects generated by pharmacological treatments.[8]

Phenolic compounds (PCs), found in plants, are an integral part of a "normal" human diet. Their daily intake depends on the diet, but is commonly evaluated at around 1 g day^{-1} for people who eat several fruits and vegetables.[9] PCs could be appropriate tools contributing to preventing the development of inflammatory diseases, more particularly in the gastro-intestinal tract, where their concentration may reach levels of up to several hundred μM.[9] Many studies have indeed reported on anti-inflammatory properties of different PCs (see ref. 10–13 for reviews).

The pomegranate (*Punica granatum* L.) originated from the Middle East is now widely cultivated and consumed throughout the world. Pomegranate has already been used for centuries in folk medicine and an explosion of interest in pomegranate as a medicinal and nutritional product has appeared within the last decade.[14–16] Numerous studies have highlighted the very high antioxidant activity of commercial pomegranate juice, which can be attributed to its high content in PCs including ellagitannins (ETs).[17–19] The beneficial health properties of pomegranates have been attributed to these ETs, like effects against cardiovascular diseases, antimicrobial and prebiotic effects, anticancer activities, and anti-inflammatory effects.[14,16,20,21] The most abundant ET found in pomegranate juice is punicalagin (PUNI), which partially migrates from the pomegranate fruit husk (PomH) during juice production.[22] PUNI is naturally found in its α- and β-anomer forms, at a concentration of about 10.5 g kg^{-1} of dry matter of pomegranate fruit husk, and at levels that can be superior to 2 g L^{-1} in industrial juice.[14,23–26] Free ellagic acid (EA) is another well-known PC found in pomegranate,[24] whereas it is more classically encountered as various esters in nature.[27]

Since a few years ago and as recently reviewed,[28] an increasing number of *in vitro* and *in vivo* studies have focused on the intestinal anti-inflammatory effects of pomegranate PC consumption. Some investigations have been conducted at the intestinal level on the effects of pomegranate PCs on cyto/chemokine gene transcription and protein secretion. Among the *in vitro* studies, a 48 h exposure of inflamed differentiated Caco-2 cells to 50 μM EA was found to slightly decrease MCP-1 and IL-8 secretion, but neither IL-6 secretion, nor IL-6, IL-8 and MCP-1 mRNA expression. Nevertheless, EA down-regulated the transcription of the IL-1 receptor type 1 gene.[29] A 4 h pre-treatment with pomegranate fruit husk extract at 50 μM in gallic acid equivalents (GAE) was shown to significantly decrease IL-8 secretion in confluent Caco-2 cells after a 48 h period of concomitant exposure with IL-1β and pomegranate.[30] In KU812 cells, a model of human mast cells, pomegranate fruit extract (20–100 μg ml^{-1}) was shown to inhibit IL-6 and IL-8 secretions and to decrease the corresponding gene transcription in a dose-dependent manner after a 1 h pomegranate pre-treatment before concomitant stimulation with phorbol 12-myristate 13-acetate and calcium ionophore.[31] In activated murine splenic CD4 + T cells, the secretion and the mRNA levels of the pro-inflammatory IL-2 gene were decreased upon 24 h exposure to 5 μM PUNI.[32]

The pomegranate biological activity and the activity of EA against intestinal inflammation have already been proven by *in vivo* studies in experimental murine models with IBD induced by sodium dextran sulphate or trinitrobenzene sulfonic acid administration.[33–36] However, no specific data have been reported on cyto/chemokine secretion or gene expression at the intestine level.

The present study was focused on the evaluation of potential anti-inflammatory properties of a PomH extract as well as of PUNI alone in an *in vitro* model of human intestine. First, PCs found in the PomH extract were identified and the PUNI and EA contents were quantified by using HPLC-DAD and LC-ESI-HRMS techniques. Differentiated Caco-2 cells cultivated in bicameral inserts were used to mimic an intestinal monolayer separating the apical compartment representative to the intestinal lumen, from the basolateral compartment representative to the underlying *lamina propria*. Among the *in vitro* intestinal cell culture models, the Caco-2 cell line is the most widely used and the best characterized system. Derived from a human colonic adenocarcinoma, these cells differentiate spontaneously into polarized intestinal cells exhibiting many properties of the small intestinal epithelium. They indeed form an apical brush border with typical small intestinal enzymes and transporters, and they exhibit tight junctions between adjacent cells.[37] Inflammation was experimentally induced by using a mixture of cytokines (IL-1β, TNF-α and IFN-γ) and LPS,[38] which significantly increases the secretion of cyto/chemokines in both apical and basolateral compartments, and induces the expression of pro-inflammatory genes.[29] The effects of PomH extract and PUNI treatments were investigated on the pro-inflammatory cytokine, IL-6 and on the chemokines IL-8 and MCP-1. The effects of the PC treatments have been first evaluated on the transcription level of the genes encoding for these 3 proteins both in normal and inflamed conditions of the intestinal mucosa. Second, IL-6, IL-8 and MCP-1 secreted in normal and inflamed conditions with or without PC treatments were quantified on both sides of the intestinal monolayer. Finally, the possible interactions between secreted cyto/chemokines and some PCs found in the PomH extract were investigated through the use of mixtures of IL-6, IL-8 or MCP-1 and PUNI in cell-free conditions.

Results

PomH extract characterization

The PomH extract under investigation for its potential anti-inflammatory properties was first submitted to a characterization of its major PCs. A 1 mg ml^{-1} solution in HBSS was analyzed by HPLC-DAD. The chromatogram obtained at 280 nm is illustrated in Fig. 1. A PUNI standard curve was built with pure

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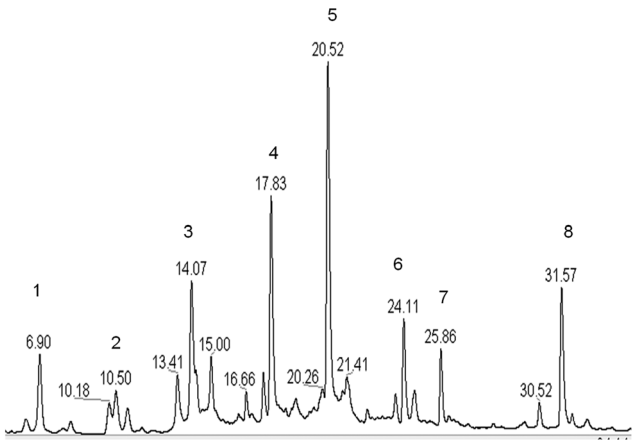

Fig. 1 Pomegranate fruit husk (PomH) extract (1 mg ml^{-1} HBSS) HPLC chromatogram at 280 nm. See Table 1 for peak assignments.

PUNI (limits of quantification (LOQ) and detection (LOD) for PUNI were respectively 3.1 nM and 0.9 nM). The PUNI concentration in the PomH extract was 80.6 ± 3.5 μM, corresponding to 87.4 ± 3.8 mg g^{-1} extract. A EA standard curve was built with pure EA (LOQ and LOD for EA were respectively 17.2 nM and 5.2 nM). The EA concentration in the PomH extract was 79.2 ± 8.4 μM, corresponding to 23.9 ± 2.5 mg g^{-1} of extract.

The identification of other PCs present in the PomH extract was made by using a LC-ESI-HRMS system in the negative mode. PUNI and EA were also injected as standards. The α- and β-anomers of PUNI[23] gave ions at *m/z* 1,083, which were verified to correspond to peak 4 and 5 (retention times of 17.83 and 20.52 min), whereas EA, which gave ions at *m/z* 301, corresponded to peak 8 (retention time of 31.57 min) on Fig. 1. Five other PCs were identified with a mass error below 4 ppm: gallic acid ([M − H]$^-$ *m/z* 169, peak 1), punicalin ([M − H]$^-$ *m/z* 781, peak 2), pedunculagin I ([M − H]$^-$ *m/z* 783, peak 3), strictinin ([M − H]$^-$ *m/z* 633, peak 6) and EA-hexoside ([M − H]$^-$ *m/z* 463, peak 7). The results are presented in Table 1.

Cytotoxic effects of the treatments on differentiated Caco-2 cells

Differentiated Caco-2 cells in bicameral inserts were exposed to PUNI and PomH treatments, 1 h before being concomitantly stimulated or not by the pro-inflammatory cocktail (IL-1β, TNF-α, IFN-γ, and LPS) during 24 h. Cytotoxicity (%) was evaluated by measuring the lactate dehydrogenase (LDH) activity in the apical and basolateral media in comparison to a positive control (full cell lysis by 1% (v/v) Triton X-100). Inflamed cells were compared to control cells. PUNI and PomH treatments in normal or inflamed conditions were compared to untreated normal or inflamed cells, respectively. No significant increase in LDH activity was detected in any case with less than 8% of LDH activity released after 24h of exposure (data not shown). The effects of the different treatments on the Caco-2 cell barrier integrity were also evaluated through the measurement of the transepithelial electrical resistance (TEER), which quantifies the movement of ions through the paracellular pathway. TEER in untreated cells was of 368 ± 23 Ω cm^2 and considered as the negative control. No significant effects on TEER values were observed neither after a 24 h exposure with the pro-inflammatory cocktail (94 ± 5%) nor upon exposure of the cells to PUNI or PomH treatments in normal (*i.e.*, 89 ± 5% and 93 ± 5%) or in inflamed conditions (*i.e.*, 96 ± 6% and 96 ± 5%), as compared to the negative control. Furthermore, no morphological alteration was detected by phase contrast microscopy.

Effects of PUNI and PomH extract on gene transcription

Normal conditions. In order to evaluate if 50 μM PUNI or 0.1 mg PomH extract ml^{-1} could modify the transcription level of the genes encoding for IL-6, IL-8 and MCP-1, non-inflamed differentiated Caco-2 cells in bicameral inserts were treated or not with PCs in the apical side during 25 h and the transcript levels were determined by qRT-PCR. Gene expression values were normalized to GAPDH RNA and expressed in fold ratio of the control (untreated cells). As shown in Table 2, no significant effects were observed.

Inflamed conditions. To mimic an inflamed intestine, differentiated Caco-2 cells in bicameral inserts, were stimulated by the addition of a mixture of cytokines (IL-1β, 25 μg l^{-1}; TNF-α, 50 μg l^{-1}; IFN-γ, 50 μg l^{-1}) to their basolateral pole and of LPS (1 mg l^{-1}) to their apical one.[38] After 24h of exposure, total RNA was isolated. qRT-PCR allowed to verify that the pro-inflammatory cocktail significantly up-regulated the transcription of the IL-6, IL-8 and CCL-2 genes by a factor of 2.1, 3.1 and 4.9, respectively (Table 2).

In order to evaluate the effects of PUNI and PomH extract, the Caco-2 cells were pre-incubated with the PCs added at the apical side during 1 h before being inflamed. After 24 h of concomitant exposure to the pro-inflammatory cocktail and PCs, total RNA

Table 1 Phenolic compounds identified in the pomegranate fruit husk (PomH) extract in full MS (scan range *m/z* 100–1500) and recorded in negative ion mode analyzed by LC-ESI-HRMS. Identification of PUNI and EA were also confirmed by injection of standard solutions

Peak	RT (min)	[M − H] *m/z*	Chemical formula	Mass error (ppm)	Compound name
1	6.90	169.01370	$C_7H_5O_5$	3.26	Gallic acid
2	10.50	781.04888	$C_{34}H_{21}O_{22}$	−3.85	Punicalin
3	14.07	783.06482	$C_{34}H_{23}O_{22}$	−3.48	Pedunculagin I
4	17.83	1083.05383	$C_{48}H_{27}O_{30}$	−4.00	Punicalagin
5	20.52	1083.05298	$C_{48}H_{27}O_{30}$	−5.19	Punicalagin
6	24.11	633.07025	$C_{27}H_{21}O_{18}$	−3.14	Strictinin
7	25.86	463.04975	$C_{20}H_{15}O_{13}$	−2.11	Ellagic acid-hexoside
8	31.57	300.99747	$C_{14}H_5O_8$	−1.41	Ellagic acid

Table 2 Relative gene transcription levels of Caco-2 cells cultivated in inserts and stimulated or not by exposure to a pro-inflammatory cocktail upon exposure or not to punicalagin (PUNI) or a pomegranate fruit husk (PomH) extract. The cells were pre-incubated or not for 1 h with the phenolic treatments (initial concentrations of 50 μM PUNI) or 0.1 mg PomH extract ml^{-1} (*i.e.*, 8 μM of PUNI)) at their apical side before being concomitantly exposed (or not) during 24h to a mixture of cytokines (IL-1β, 25 $\mu g\ l^{-1}$; TNF-α, 50 $\mu g\ l^{-1}$; IFN-γ, 50 $\mu g\ l^{-1}$) at their basolateral side and to LPS (1 $mg\ l^{-1}$) at their apical side

		PUNI		PomH extract	
Ratio[a]	Pro-inflammatory cocktail[b]	Normal[b]	Inflamed[c]	Normal[b]	Inflamed[c]
IL-6 mRNA	**2.09 ± 0.30****	1.13 ± 0.35 (p > 0.1)	**0.44 ± 0.13****	1.24 ± 0.19 (p > 0.1)	**0.22 ± 0.08*****
IL-8 mRNA	**3.09 ± 0.63***	1.33 ± 0.28 (p > 0.1)	0.71 ± 0.07 (p = 0.01)	1.24 ± 0.40 (p > 0.1)	0.62 ± 0.22 (p > 0.1)
MCP-1 mRNA	**4.88 ± 0.82****	0.77 ± 0.13 (p > 0.1)	**0.45 ± 0.10*****	1.38 ± 0.20 (p > 0.1)	**0.27 ± 0.05*****

[a] Results are means ± SEM, $n = 3$, bold-faced values are considered significant, as both the p-values are below 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and the ratios are either above 1.5 (up-regulation) or below 0.5 (down-regulation). [b] mRNA levels relative to the non-inflamed control group. [c] mRNA levels relative to the inflamed control group.

was extracted and qRT-PCR performed. Both PUNI and the PomH extract significantly down-regulated IL-6 and MCP-1 transcription with greater effects of the PomH extract, while no effects were observed on IL-8 gene transcription (Table 2).

Effects of PUNI or PomH extract on the secretion of cyto/chemokines

Effects on apical and basolateral sides. After a 1 h treatment with PUNI or PomH extract in the apical compartment at initial concentrations of 50 μM and 0.1 $mg\ ml^{-1}$, respectively, cells were further treated (or not) in the basolateral side with the mixture of cytokines and in the apical side with LPS, still in the presence of the PCs during a 24 h period. The amounts of IL-6, IL-8 and MCP-1 secreted in the apical and basolateral sides were then quantified by ELISA.

In the absence of the pro-inflammatory cocktail, the values were below the limit of detection for IL-6 (LOD of 2.2 $pg\ ml^{-1}$) at both the apical and basolateral sides. The same was true for MCP-1 at the basolateral side (LOD of 1.0 $pg\ ml^{-1}$). By contrast, IL-8 could be quantified at both sides (LOD of 0.8 $pg\ ml^{-1}$). In terms of quantities secreted (Table 3), the values were 3 times higher at the apical medium.

Upon exposure to the pro-inflammatory cocktail (Fig. 2), the secretion of the 3 cyto/chemokines was significantly enhanced both at the apical and basolateral sides. Fold increases could be calculated for IL-8 at both sides (3-fold at the apical side and 41-fold at the basolateral side) and for MCP-1 at the apical side (5-fold). The secretion increased thus in both apical and basolateral sides upon exposure to the pro-inflammatory cocktail, but, at least for IL-8 and MCP-1, this increase was more important at the basolateral side than at the apical one.

Table 3 shows the results upon exposure to initial concentrations of PUNI at 50 μM or PomH extract at 0.1 $mg\ ml^{-1}$ in the absence of pro-inflammatory stimuli. Results for IL-6 remained below the LOD. The IL-8 concentration significantly decreased with PUNI and PomH extract treatments in the apical media, while no significant effect was observed in the basolateral media. The same effects were observed for MCP-1 concentration with both treatments in the apical media while the concentrations in the basolateral media remained under the LOD.

As shown in Fig. 2, in inflamed Caco-2 cells, upon exposure to PUNI or PomH extract, IL-6, IL-8 and MCP-1 concentrations significantly decreased ($p < 0.001$) in the apical compartments, when compared to the inflamed untreated controls. However, the decrease was quantitatively limited for IL-8, in comparison to IL-6 and MCP-1. Higher effects were observed for IL-6 and for MCP-1 than for IL-8.

Considering that 100% represented the apical side of the inflamed cells, IL-6 indeed decreased by 66% with PUNI and by 74% with the PomH extract, and MCP-1 decreased by 82% with PUNI and by 83% with the PomH extract, while IL-8 decreased only by 26% with PUNI and by 36% with the PomH extract. No significant differences were observed between both treatments.

Table 3 Effects of punicalagin (PUNI) and of the pomegranate fruit husk (PomH) extract on IL-6, IL-8 and MCP-1 secretion by differentiated Caco-2 cells in inserts in non-inflamed conditions. 21 day post-confluent Caco-2 cells, cultivated in bicameral inserts, were exposed to either PUNI (initial concentration of 50 μM) or PomH extract (0.1 $mg\ ml^{-1}$) at the apical compartment. After 25 h of exposure, apical and basolateral media were separately collected and centrifuged before quantifying IL-6, IL-8 and MCP-1 levels by ELISA. Results were compared to those obtained in control cells. Control cells were not submitted to any treatment. Values represent means ± SEM, $n = 3$, and are expressed in pg. Asterisks indicate a significant difference as compared to the control cells after ANOVA post-Hoc Tukey analyses. The limits of detection were respectively 2.2 $pg\ ml^{-1}$ for IL-6, 0.8 $pg\ ml^{-1}$ for IL-8 and 1.0 $pg\ ml^{-1}$ for MCP-1. The volume of apical and basolateral compartments were 0.5 and 1.5 ml, respectively

	Control cells		Punicalagin		PomH extract	
Cytokine [pg]	Apical	Baso-lateral	Apical	Baso-lateral	Apical	Baso-lateral
IL-6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
IL-8	16.8 ± 2.1	5.4 ± 0.6	5.9 ± 0.7[a]	4.8 ± 0.2	4.0 ± 0.4[a]	5.3 ± 0.2
MCP-1	2.3 ± 0.2	<LOD	<LOD	<LOD	0.6 ± 0.0[a]	<LOD

[a] $p < 0.001$.

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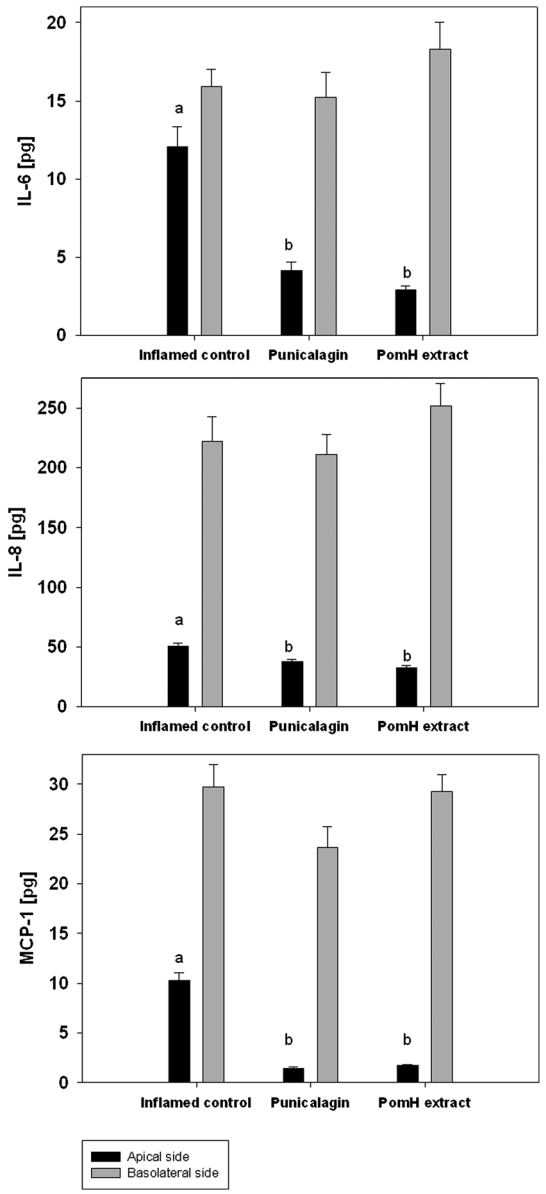

Fig. 2 Effects of punicalagin (PUNI) and of the pomegranate fruit husk (PomH) extract on IL-6, IL-8 and MCP-1 secretion by differentiated Caco-2 cells in inserts, under inflamed conditions. 21-day post-confluent Caco-2 cells, cultivated in bicameral inserts, were pretreated with PUNI (initial concentration of 50 μM) or the PomH extract (0.1 mg ml^{-1}) in the apical compartment during 1 h before being exposed at the basolateral side to a mixture of cytokines (IL-1β, 25 μg l^{-1}; TNF-α, 50 μg l^{-1}; IFN-γ, 50 μg l^{-1}) and at the apical side with LPS (1 mg l^{-1}) during 24 h still in the presence of the treatments. IL-6, IL-8 and MCP-1 of the apical and basolateral compartments were quantified by ELISA and compared to the inflamed untreated control. Letters indicate significant differences ($p < 0.001$) after ANOVA post-Hoc Tukey analysis. Results are means ± SEM, $n = 3$.

However, treatments with the PomH extract corresponded to a concentration of about 8 μM PUNI and seemed therefore more potent than PUNI alone. Concerning the basolateral side, no significant effects of PUNI and PomH extract were observed for IL-6, IL-8 and MCP-1, as compared to the cyto/chemokine content of the basolateral medium for the inflamed control.

Dose-response experiments. After a 1 h pre-treatment with increasing doses of PUNI ranging from 0.1 to 100 μM or of PomH extract ranging from 0.02 to 0.50 mg ml^{-1} (corresponding to initial concentrations of PUNI of 1.3 to 42.4 μM), differentiated Caco-2 cells were treated with the same pro-inflammatory cocktail as above, still in the presence of the treatments. The amount of IL-6, IL-8 and MCP-1 secreted in the extracellular media was then quantified by ELISA. Results are shown in Fig. 3.

Upon incubation with PUNI, no effects were observed with the concentrations from 0.1 μM to 10 μM on IL-6 secretion. PUNI at the concentration of 25 μM decreased significantly the IL-6 secretion to 46%, when 100% represented the positive control (inflamed cells). The higher concentrations also significantly decreased the IL-6 secretion reaching 26% of positive control for 100 μM of PUNI. No anti-inflammatory effects were observed ≤5 μM of PUNI on IL-8 secretion, but the effects became significant at 10 μM of punicalagin, where IL-8 secretion decreased to 80% of the inflamed control; effects were higher with increased concentrations to reach 20% of the inflamed control with a punicalagin concentration of 100 μM. No anti-inflammatory effects were observed ≤10 μM of PUNI on MCP-1 secretion, but the effects became significant at 25 μM of PUNI, where MCP-1 secretion significantly decreased to 39% of the inflamed control. The same dose effects were observed for MCP-1 secretion than for IL-6 and IL-8 reaching 23% of the inflamed control with 100 μM of PUNI.

Regarding the effects of PomH extract treatments, no effects were observed with the concentrations of PUNI-contained PomH extract from 1.3 μM to 5.3 μM on IL-6 secretion. IL-6 secretion was significantly decreased to 35% of the inflamed control from 10.6 μM of PUNI-contained PomH extract (*i.e.*, 0.13 mg ml^{-1} PomH extract). Anti-inflammatory effects on IL-8 secretion were already significant from 5.3 μM of PUNI-contained PomH extract (*i.e.*, 0.07 mg ml^{-1} PomH extract) reaching 54% of the inflamed control. For MCP-1 secretion, a significant decrease was also observed from 5.3 μM of PUNI-contained PomH extract to 60% of positive control was observed from 3.4 μM punicalagin-contained PomH extract ($p < 0.01$). As observed for treatments with pure PUNI, PomH extract treatments decrease IL-6, IL-8 and MCP-1 secretion in inflamed Caco-2 cells in a dose-dependent manner, but these effects are more powerful effects than those observed with PUNI alone.

Direct interaction between PUNI and cyto/chemokines

To evaluate a possible direct molecular trapping of the three pro-inflammatory cyto/chemokines by the PC treatments after their secretion in the culture medium of Caco-2 cells, increasing concentrations of PUNI, ranging from 1 to 100 μM, were set in multiwells before adding constant concentrations of IL-6 (14.3 pg ml^{-1}), IL-8 (9.3 pg ml^{-1}), or MCP-1 (29.7 pg ml^{-1}). After a 24 h incubation period, these 3 cyto/chemokines were quantified in each well by ELISA and compared to the respective control, *i.e.*, the same concentration of cyto/chemokines without PUNI. The amount of immuno-detected cyto/chemokines significantly

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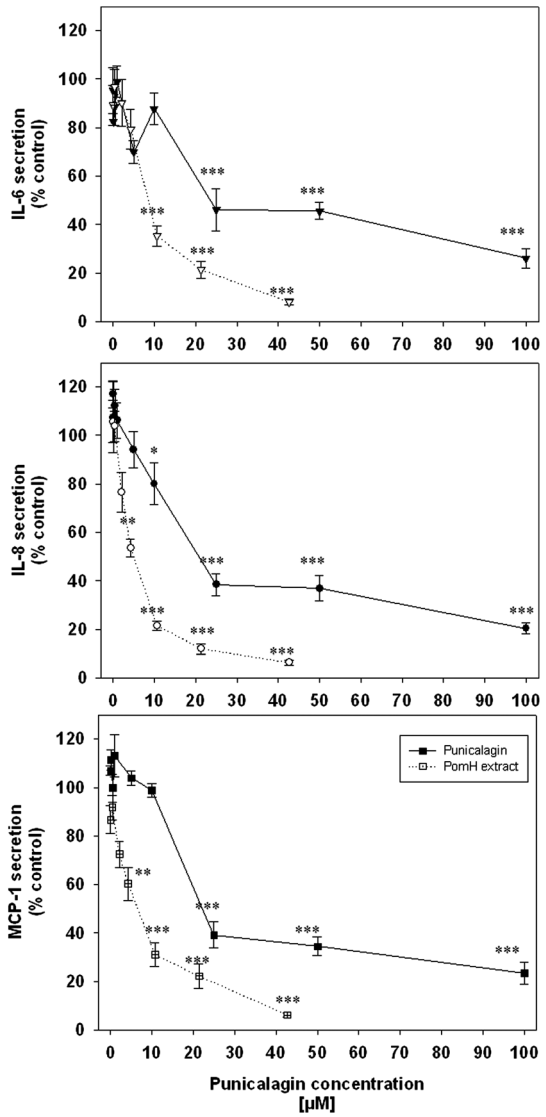

Fig. 3 Effects of increasing doses of punicalagin (PUNI) (solid line), or of PUNI-contained in PomH extracts (dotted line), on IL-6 (triangle), IL-8 (circle) and MCP-1 (square) secretion by differentiated Caco-2 cells in wells, under inflamed conditions. 21-Day post-confluent Caco-2 cells, cultivated in wells, were pretreated with increasing initial concentrations of (0.1 to 100 μM) or of PomH extract (0.02 to 0.5 mg ml^{-1}; *i.e.*, initial concentrations of PUNI of 1.3 to 42.4 μM.). After 1 h of preincubation, inflammation was induced with a mixture of cytokines (IL-1β, 25 μg l^{-1}; TNF-α, 50 μg l^{-1}; IFN-γ, 50 μg l^{-1}) and LPS (1 mg l^{-1}) during 24 h still in the presence of the treatments. IL-6, IL-8 and MCP-1 were quantified by ELISA in the extracellular media and compared to the inflamed untreated control. Asterisks indicate significant differences (*$p < 0.05$,**$p < 0.01$ and ***$p < 0.001$) after ANOVA followed by Dunnett's post-hoc multiple range tests analysis. Results are means ± SEM, $n = 4$.

decreased from 10 μM PUNI for IL-6 and from 1 μM PUNI for IL-8 and MCP-1. Higher effects were observed when PUNI concentration increased, as shown in Fig. 4.

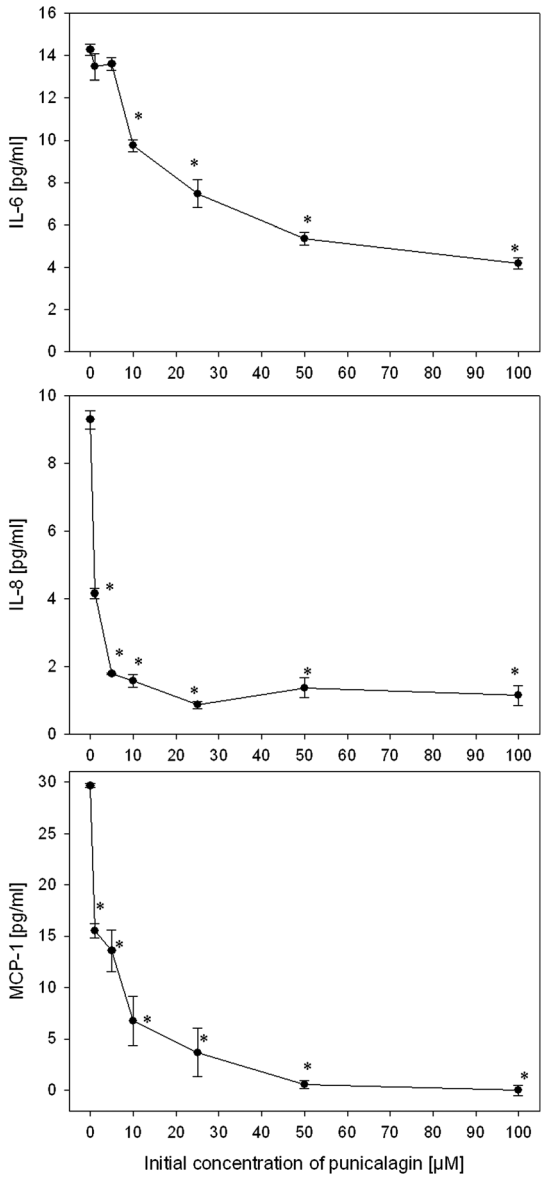

Fig. 4 Interactive effects between punicalagin (PUNI) and IL-6, IL-8 and MCP-1 Increasing concentrations of PUNI, prepared in a cell-free culture medium, were incubated in 12 well-plates during 1h before adding constant concentration of either IL-6 (14.3 pg ml^{-1}), IL-8 (9.3 pg ml^{-1}), or MCP-1 (29.7 pg ml^{-1}). After a 24 h incubation period, media were collected and centrifuged. Cyto/chemokines were quantified by ELISA and compared to the respective control without PUNI (*i.e.*, cell-free culture medium with the same concentration of cyto/chemokines). An asterisk indicates a significant difference ($p < 0.001$) after ANOVA and Dunnett's post-hoc analyses. Results are means ± SEM, $n = 3$.

Discussion

The intestinal production of pro-inflammatory cyto/chemokines (*e.g.*, IL-6, IL-8 and MCP-1) is a physiological and vital event, which aims at activating the mucosal immune and intestinal epithelial cells in acute situations. This process may become chronic, as it is the case *e.g.*, during IBDs. IL-6 is a pleiotropic cytokine acting as a regulator of acute phase responses and a lymphocyte-stimulatory factor. IL-8 and MCP-1 are

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chemokines with chemoattractant and pro-inflammatory functions: IL-8 attracts neutrophils, but not monocytes, while MCP-1 attracts monocytes (but not neutrophils) on the sites of inflammation.[39–41]

ETs were identified as the main PCs in the PomH extract used in this study with PUNI and EA as major compounds. EA was, however, not considered separately in our *in vitro* experimental model, because of its very poor solubility in the culture medium,[42] as well as the anti-inflammatory properties attributed to its classical solvent (*i.e.*, dimethyl sulfoxide).[43] In this study, we evaluated the effects of treatments with the PomH extract and PUNI, as reference, on IL-6, IL-8 and MCP-1 in both control ("physiological") culture conditions of Caco-2 cells and upon exposure to a pro-inflammatory cocktail (IL-1β, TNF-α and IFN-γ and LPS[38]), which increased significantly the secretion of the 3 cyto/chemokines, as well as up-regulated the transcription of the 3 genes.[29]

In a first step, the effects of a given concentration of PUNI (*i.e.*, 50 μM) or PomH extract (0.1 mg ml^{-1}, corresponding to a concentration of 8 μM PUNI) were evaluated on the transcription levels of the genes encoding IL-6, IL-8 and MCP-1, both in "physiological" and "inflamed" culture conditions, in Caco-2 cells grown in bicameral inserts. The apical compartment, representative of the intestinal lumen, was exposed to PC treatments, while the basolateral one, representative of the *lamina propria*, received or not the mixture of cytokines inducing the inflammation (except for LPS added in the apical compartment). In our study, no significant down-regulation was observed in normal conditions on IL-6, IL-8 or MCP-1 gene transcription, as well as on IL-8 in inflamed conditions, whereas the transcription of IL-6 and MCP-1 genes upon pro-inflammatory stimulation was down-regulated with both treatments.

Only a small amount of data is available on the effects of pomegranate consumption on IL-6, IL-8 and MCP-1 gene transcription in the literature.[29,31] Haqqi *et al.* showed a similar decrease as we did on the IL-6 gene transcription in a dose-dependent manner after a 1 h pomegranate pre-treatment before concomitant stimulation with phorbol-12-myristate 13-acetate (PMA) plus calcium ionophore A23187 (CI) in KU812 cells, a model of human mast cells involved in intestinal inflammatory events.[31] This group also observed a significant decrease of IL-8 gene transcription, whereas the results were not significant in our case, even though a tendency was visible. As for MCP-1, the present study is, to our knowledge, the first work describing a down-regulation of MCP-1 gene transcription by a PomH extract and by PUNI. PUNI may not be the only active compound in PomH, with regard to decrease of cyto/chemokine gene transcription. Similar effects are indeed obtained with 50 μM PUNI and with the PomH extract offering a PUNI concentration of only 8 μM. The additional effect with the PomH extract might, however, not be due to the presence of EA, which is one of the major PCs identified in this PomH extract, since a concentration of 50 μM EA was reported not to have an effect on IL-6, IL-8 and MCP-1 transcription on inflamed Caco-2 cells.[29]

In a second step, the IL-6, IL-8 and MCP-1 proteins secreted in normal and inflamed conditions by Caco-2 cells, were quantified separately in both compartments of the bicameral inserts. When the intestinal cells were stimulated basolaterally with the mixture of cytokines and apically with LPS, IL-6, IL-8 and MCP-1 were released in both the apical and basolateral directions. These results confirm the capability of intestinal Caco-2 cells to respond to pro-inflammatory stimuli, but, interestingly, they indicate that, at least for IL-8 and MCP-1, the fold increases were much higher in the basolateral compartment. Surprisingly, the PUNI and PomH extract treatments led to decreased concentrations of the three cyto/chemokines, but only in the apical compartment, while no significant effect was observed in the basolateral one. The effects on the apical sides were significant for IL-6, IL-8 and MCP-1 but less pronounced for IL-8. A previous study performed in our lab with confluent Caco-2 cells in multiwell plates indicated that 50 μM GAE of a PomH extract significantly decreased IL-8 secretion in inflamed conditions.[30] IL-6 and MCP-1 were however unfortunately not looked at. Another pomegranate fruit extract was found to decrease IL-6 and IL-8 secretion in a dose-dependent manner in stimulated human mast cells.[31] Pomegranate juice consumption was also shown to decrease IL-6 secretion in prostate cancer cells reducing inflammation and its impact on tumor cells proliferation.[44] Regarding individual compounds found in the PomH extract used in the present study, only few data are available on the cyto/chemokines secretion and gene transcription. EA was reported to slightly decrease IL-8 and MCP-1 but not IL-6 secretion in differentiated Caco-2 cells under a pro-inflammatory stimulation.[29] EA was also reported to decrease MCP-1 levels in the culture supernatant of IL-1β or TNF-α stimulated pancreatic stellate cells.[45] Gallic acid was also reported to decrease IL-6 secretion and gene expression in PMA-CI stimulated human mast cells,[46] as well as IL-6 and IL-8 gene expression and secretion in *Fusobacterium nucleatum*-activated oral epithelial cells.[47]

Regarding the effects on IL-6, IL-8 and MCP-1 secretion in *in vivo* models, no data were found at the intestinal level. However, an *in vivo* study on collagen-induced arthritis in mice examined the effects of dietary supplements with pomegranate extract on the joint inflammation associated with this disease. The IL-6 levels in arthritic joints of pomegranate supplemented mice significantly decreased compared to those without any supplementation and these effects were more effective at higher doses.[48] A topical application of EA decreased IL-6 secretion in skin tissues of UV-B-irradiated mice.[49]

In addition to the effects on the cyto/chemokines, other anti-inflammatory effects of the pomegranate, its individual ETs, as well as its metabolites urolithins have been reported, both in *in vitro* and *in vivo* models, by acting on other mechanisms involved in the inflammatory response (see ref. 14,15 for reviews).

Clinical studies with humans reported divergent effects of pomegranate consumption on IL-6 secretion. A study on healthy volunteers submitted to an eccentric exercise provoking a local inflammation, showed that a pomegranate extract supplement allowed to improve strength recovery, but had no effect on the serum IL-6 concentration.[50] The inflammation marker IL-6 was also unaffected in the plasma of healthy volunteers 4 h after an acute consumption of pomegranate extract.[51] By contrast, patients with periodontitis receiving subgingival chips impregnated with pomegranate extract (and *Centella asiatica* extract) presented lower IL-6 concentrations in the gingival cervicular fluid several months after the treatment.[52]

Regarding the intracellular mechanisms, PCs of pomegranate seem to act on 2 major intracellular signal transduction cascades of inflammation, since pomegranate husk PCs were shown to inhibit NF-κB and MAPK cascades in an *in vitro* model of inflamed intestinal cells[30] and mastocytes,[31] as well as *in vivo* in rats.[36]

Finally, possible interactions between secreted cyto/chemokines and PC treatments were investigated. Pure (*i.e.*, purchased from a commercial source) IL-6, IL-8 or MCP-1 were incubated with concentrations of PUNI (ranging from 1 to 100 μM) in the same experimental conditions as for the cell experiments. The concentration of the three pro-inflammatory cyto/chemokines, as detected by ELISA, decreased in a dose-response manner when PUNI treatments were applied. This observation strongly suggests a direct interaction effect between this ET and the pro-inflammatory cytokine. ETs, the main PCs identified in the PomH extract, are known to interact with substances like proteins.[53] However, to our knowledge, it is the first time that interactions between ETs and pro-inflammatory cytokines are reported. Such interactions in the intestinal lumen may have a physio-pathological significance. Indeed, the presence of cytokines in stools of patients with IBDs suggests the secretion of pro-inflammatory cytokines in the intestinal lumen.[54] Moreover, a paracrine communication role of the proinflammatory cytokines *via* the gut lumen has been recently suggested by Sonnier *et al.*[55] Considering the central role of cyto/chemokines in influencing/regulating the intestinal inflammatory responses, the highlighted interactions between the three pro-inflammatory cyto/chemokines (IL-6, IL-8 and MCP-1) and PUNI,the PomH ETs could play an important role in IBDs. In other words, PomH ETs could interact with the pro-inflammatory cytokines in the gut lumen, decreasing in turn the intercellular communication, and limiting the mediation of the local and systemic inflammation. The trapping effect might thus be added to the effects on the transcript levels of IL-6 and MCP-1, and lead to complementary effects in terms of inflammatory modulation.

Further studies should be conducted to identify the important players in the interactions between PomH ETs and cyto/chemokines. It is generally admitted that proline plays an important role in the interaction of protein with tannins.[56] The percentages of proline found in the three cyto/chemokines are respectively of 3% for IL-6, 5% for IL-8, and 6% for MCP-1 (GenBank accession numbers are as follows: AAC41704.1 (Human IL-6); AAH13615 (Human IL-8); AAB20651.1 (Human MCP-1)). It may be interesting to evaluate if these differences in proline percentage influence the interactions with tannins. Considering the possible physico-chemical interactions between ETs and cyto/chemokines, and the potential discrepancy between mRNA transcription and protein level, it would also be of interest to measure the suspected changes in cyto/chemokine protein neosynthesis through the incubation of Caco-2 cells with labeled amino acids followed by immunoprecipitation.

Experimental

Chemicals and samples

PUNI (95.35% purity) was purchased from Phytolab (Vestenbergsgreuth, DE). EA (>90% purity) was purchased from Extrasynthese (Lyon, FR). Human recombinant IL-1β (>98% purity), TNFα (>98% purity) and LPS were purchased from Sigma-Aldrich (St Louis, MO) and IFN-γ (≥98% purity) was from Calbiochem (Darmstadt, DE). Acetonitrile and trifluoroacetic acid for liquid chromatography analyses were from Biosolve (Valkenswaard, NL). An hydroalcoholic extract of Pomegranate (*Punica granatum* L.) husk (PomH extract) was prepared from Moroccan cultivars and provided by Stiernon (Ath-Ghislenghien, BE) in the form of a dry powder.

Cell culture

Caco-2 cells (passage 27–37) were obtained from the American Type Culture Collection (ATCC number HTB-37, LGC Promochem, Molsheim, FR). They were routinely cultivated in 75 cm^2 flasks (Corning CellBIND®, Badhoevedorp, NL) in standard Dulbecco's modified Eagle's medium (DMEM), containing 25 mM glucose, 4 mM glutamine and supplemented with 0.1% (v/v) non essential amino acids (Invitrogen, Merelbeke, BE) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Perbio Science, Erembodegem, BE). Medium was changed 3 times a week and cells were incubated at 37 °C in a humidified atmosphere of air/carbon dioxide (95 : 5, v/v).

Cell treatments

Caco-2 cells (passage 27–37) were harvested at 90% of confluence by using 0.05% Trypsin-0.02% EDTA (Lonza, Walkersville, MD) and seeded on 12-insert plates (Corning Transwell®) or on 12-well plates (Corning CellBIND®) with a density of 2.10^4 cells cm^{-2}. Cells were cultivated in standard medium for 21 days post-confluence. The day of experiment, the medium was removed and cells washed twice with DMEM supplemented with 0.5% FBS and without phenol red.

For experiments in inserts, differentiated Caco-2 cells were preincubated for a 1h period, with PUNI at 50 μM or with the PomH extract at 0.1 mg ml^{-1} (corresponding to 8 μM of PUNI) in the apical compartment of the cells. PUNI was solubilized in 25% MeOH (v/v) at 50 mM, and added to the culture medium at the final concentration of 50 μM. The PomH extract was solubilized in Hank's balanced saline solution (HBSS) (BD Biosciences Pharmingen, San Diego, CA) at the concentration of 1 mg ml^{-1} and added to the culture medium at the final concentration of 0.1 mg ml^{-1}.

For experiments in wells, differentiated Caco-2 cells were were preincubated for a 1h period with increasing concentrations of PUNI from 0.1 to 100 μM or with increasing concentrations of PomH extract from 0.02 to 0.5 mg ml^{-1} corresponding to PUNI concentrations from 1.3 to 42.4 μM. After 1 h of preincubation, inflammation was induced or not with a mixture of cytokines (IL-1β, 25 μg l^{-1}; TNF-α, 50 μg l^{-1}; IFN-γ, 50 μg l^{-1}) and LPS (1 mg l^{-1}),[38] which was left in contact with the cells during 24 h, still in the presence of the tested PCs. For experiments in inserts, the mixture of cytokines was introduced at the basolateral side and LPS at the apical side.

Cytotoxicity assay

The possible cytotoxicity of treatments or of the pro-inflammatory cocktail was routinely checked through the determination of

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the activity of the lactate dehydrogenase (LDH) released by the cells into the extracellular medium, with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, DE) used according to the manufacturer's instructions. The percentage of cytotoxicity was calculated using the maximum releasable LDH activity obtained upon complete lysing of the Caco-2 cells monolayers of adequate control inserts with 1% (v/v) Triton X-100 (Sigma-Aldrich) (100% LDH activity released). Furthermore, the integrity of the monolayers was checked before and after each treatment, by the measurement of the TEER (Endohm-24, World Precision Instruments, Sarasota, FL).

Transcription of IL-8, IL-6 and CCL-2

Differentiated Caco-2 cells (21 days post-confluence) in inserts were preincubated or not with the tested PomH extract or PUNI at the apical side during 1h before being stimulated or not with the pro-inflammatory cocktail as described above. Total RNA was isolated from the cell monolayer using the TRIZol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quality and quantity of total RNA were assessed by 1.5% (w/v) agarose gel electrophoresis and Nanodrop spectrophotometry. Total RNA (5 μg) was reverse-transcribed using the Superscript III First-Strand Synthesis System for quantitative real-time Polymerase Chain Reaction (qRT-PCR) (Invitrogen) following manufacturer's instructions. Then, qRT-PCR was performed in a 96 well PCR plates (Bio-Rad, Nazareth Eke, BE), to estimate the mRNA abundance corresponding to either IL-6, IL-8, or MCP-1 relatively to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used for normalization.[57] A total of 5 μl cDNA with the appropriate dilution, 0.2 μl primer mix and 4.8 μl SybrGreen SuperMix (Bio-Rad) were added to each well. The PCR amplifications were performed in a MyIQ2 thermocycler (Bio-Rad) as follows: 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Melting curves were systematically assessed for quality control. Gene expression values were normalized to the expression value of GAPDH RNA and calculated on the basis of the comparative cycle threshold (Ct) method (2 -ΔΔCt). The primers used were: IL-6 (sense 5′-ACTCCTTCTCCACAAGCGCCTTC-3′; antisense 5′-TGAAGAGGTGAGTGGCTGTC-3′); IL-8 (sense 5′-CTGGCCGTGGCTCTCTTG-3′; antisense 5′-GGGTGGAAAGGTTTGGAGTATG-3′); MCP-1 (sense 5′-TTCTCAAACTGAAGCTCGC-3′; antisense 5′-TGATTGCATCTGGCTGAGC-3′); GAPDH (sense 5′-ACCCACTCCTCCACCTTTGAC-3′; antisense 5′-GTCCACCACCCTGTTGCTGTA-3′).

Secretion of pro-inflammatory cyto/chemokines (IL-6, IL-8 and MCP-1)

After 24 h, the apical and basolateral media from inserts were separately collected and centrifuged at 3000 × *g* for 10 min as for apical media from wells. The IL-6, IL-8 and MCP-1 levels were then quantified by using, respectively, the Human IL-6, IL-8 and MCP-1 ELISA Kits (BD Biosciences Pharmingen), which are sandwich ELISA using monoclonal antibodies specific for IL-6, IL-8 or MCP-1. IL-6, IL-8 and MCP-1 were quantified using respective standard curves and the limits of detection (LOD) were considered to be 2.2, 0.8 and 1.0 pg ml^{-1}, according to the manufacturer's informations, respectively. For experiments in inserts, the results were adjusted to the total volume of the compartment since the cells were used to extract the RNA. For experiments in wells, the results were adjusted to the total cell protein content of each well, as measured with the bicinchoninic acid protein (BCA) assay Kit (Sigma-Aldrich) using the serum albumin as standard.[58] Results are expressed relatively to the appropriate positive control (control cells stimulated with the pro-inflammatory cocktail or not).

Quantification of punicalagin

The quantification of PUNI in the PomH extract was carried out with an HPLC equipement (Thermo Separation Products, San Jose, CA) consisting of a P4000 pump, equipped with an AS 100 autoinjector, a SN 4000 interface, a UV6000LP detector (ThermoFinnigan, San Jose, CA) and the Chromquest software. The analytical column consisted in a Phenomenex Gemini C18 reversed-phase column (3 μm; 150 × 4.6 mm) (Phenomenex, Torrance, CA) preceded by a 4.0 × 3.0 mm guard column of the same type. The columns were maintained at 40 °C by a temperature controller. Elution was performed with a gradient made with 0.1% trifluoroacetic acid (TFA) in water (v/v) (solvent A) and 0.1% TFA in acetonitrile (v/v) (solvent B), according to the following programme: 99% A to 70% A over 40 min; 70% A to 1% A over 15 min, 1% A to 99% A over 3 min; re-equilibration time. The volume injected was 20 μl and the flow rate was maintained at 1 ml min^{-1}. The samples were filtered through a 0.45 μm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. The wavelengths for acquisition of UV spectra were 200–600 nm. PUNI was quantified at 280 nm. For the calibration curves, a PUNI standard was prepared in 25% MeOH at the concentration of 50 mM and then diluted in the same solvent. The PomH extract was solubilized in HBSS at 1 mg ml^{-1} and passed through a 0.45 μm filter before injection.

Identification of the PomH phenolic compounds

The identification of the PCs present in the PomH extract was carried out using a liquid chromatography-electrospray ionization-high resolution mass spectrometry (LC-ESI-HRMS) system (ThermoFisher, Bremen, DE) consisting of a LTQ-Orbitrap-XL mass spectrometer equipped with an Accela (ThermoFisher, San Jose, CA) HPLC. The column was similar to the one used for the HPLC-DAD quantification, except for the internal diameter, which was 2 mm instead of 4.6 mm, and the flow rate, which was set at 200 μl min^{-1}. The gradient was also similar except that the TFA was replaced by formic acid. The following ESI conditions were applied: 5 kV of spray voltage; sheath gas (N_2) flow rate of 35 and auxiliary gas (N_2) flow rate of 10 arbitrary units; temperature of the capillary set at 275 °C; tube lens voltage of −203 V. Tuning of the ESI source was done with PUNI.

Statistical analysis

All the results were expressed as means ± SEM. For pro-inflammatory marker analyses, homogeneity of variances was previously tested by the Levene's test and data were analyzed by

one-way ANOVA followed by the Dunnett or Tukey's post-hoc multiple range tests to identify means with significant differences. For the RT-PCR analyses, Student's *t*-unpaired tests were performed to compare the values with the appropriate control (non-stimulated cells or stimulated cells). The accepted level of significance was set at a p value <0.05. All these statistical analyses were performed using Entreprise Guide 4.2 software (SAS Institute, Cary, NC).

Conclusion

In summary, the present study showed that a pomegranate fruit husk (PomH) polyphenolic extract, rich in punicalagin, present anti-inflammatory properties in an *in vitro* model of human intestine. Differentiated Caco-2 cells cultivated in bicameral inserts were pretreated or not with a PomH extract or punicalagin, as reference, at the apical side, representing the intestinal lumen, before inflammation was induced by a mixture of cytokines and LPS during 24 h still in the presence of the treatments. Cytokine and chemokine networks play an important role in the initiation and perpetuation of the inflammatory reaction in IBD. In this study the effects of the treatments were assayed on three pro-inflammatory cyto/chemokines, *i.e.*, interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1 both at their gene transcription (qRT-PCR) and secretion (ELISA) levels. The PomH extract showed anti-inflammatory properties in inflamed intestinal cells by acting both on the pro-inflammatory gene transcription (as for IL-6 and MCP-1) and protein levels (as for IL-6, IL-8 and MCP-1), the later phenomenon being possibly due to a direct molecular trapping by ellagitannins of the PomH extract. To our knowledge, it is the first time that a direct interaction between cyto/chemokines and ETs is proposed. These new results in addition to the existing data about anti-inflammatory properties of pomegranate, suggest that pomegranate fruit husk, let as by-product of the pomegranate fruit juice industry, could be an interesting natural source contributing to prevent intestinal chronic inflammation.

Abbreviations

BCA	bicinchoninic acid protein
DMEM	Dulbecco's modified eagle medium
EA	ellagic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
ET	ellagitannin
FBS	fetal bovine serum
GAE	gallic acid equivalent
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hank's balanced saline solution
HRMS	high resolution mass spectrometry
IBD	inflammatory bowel diseases
IFN	interferon
IL	interleukin
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
MCP	monocyte chemoattractant protein
PC	phenolic compound
PomH	pomegranate fruit husk
PUNI	punicalagin
qRT-PCR	quantitative real-time polymerase chain reaction
TEER	transepithelial electrical resistance
TFA	trifluoroacetic acid
TNF	tumor necrosis factor
UC	ulcerative colitis.

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