Strawberry tannins inhibit IL-8 secretion in a cell model of gastric inflammation

Marco Fumagalli a, Enrico Sangiovanni a, Urska Vrhovsek b, Stefano Piazza a, Elisa Colombo a, Mattia Gasperotti b, Fulvio Mattivi b, Emma De Fabiani a, 1, Mario Dell’Aglì a, 1

a Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Italy
b Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all’Adige, Italy

A R T I C L E   I N F O

Article history:
Received 23 June 2016
Received in revised form 22 July 2016
Accepted 22 July 2016
Available online 27 July 2016

Chemical compounds studied in this article:
Agrimoniin
Casuarictin
Ellagic acid
Procyanidin B1

Keywords:
Ellagitannins
Gastric inflammation
IL-8
Procyanidins
Strawberry
Fragaria spp

A B S T R A C T

In the present study we chemically profiled tannin–enriched extracts from strawberries and tested their biological properties in a cell model of gastric inflammation. The chemical and biological features of strawberry tannins after in vitro simulated gastric digestion were investigated as well. The anti-inflammatory activities of pure strawberry tannins were assayed to get mechanistic insights.

Tannin-enriched extracts from strawberries inhibit IL-8 secretion in TNFα-treated human gastric epithelial cells by dampening the NF-κB signaling. In vitro simulated gastric digestion slightly affected the chemical composition and the biological properties of strawberry tannins. By using pure compounds, we found that casuarictin may act as a pure NF-κB inhibitor while agrimoniin inhibits IL-8 secretion also acting on other biological targets; in our system procyanidin B1 prevents the TNFα-induced effects without interfering with the NF-κB pathway.

We conclude that strawberry tannins, even after in vitro simulated gastric digestion, exert anti-inflammatory activities at nutritionally relevant concentrations.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

The aetiopathogenesis of gastritis, an inflammatory state of gastric mucosa, is mostly due to Helicobacter pylori (H. pylori), a Gram-negative pathogen affecting humans and primates. This bacterium has been classified as a Type 1 carcinogen by WHO, thus making H. pylori-associated gastritis a relevant public health concern. The clinical outcome is influenced by both microbial pathogenicity and by host factors. Uncontrolled chronic gastritis may lead to more severe pathological conditions such as gastric and duodenal ulcers, mucosal atrophy, gastric carcinoma, or gastric lymphoma.

During H. pylori infection, gastric epithelial cells show higher levels of cytokines including IL-1β, TNFα and IL-8, a potent neutrophil-activating chemokine playing a key role in gastric diseases [1,2]. This response is highly dependent on activation of NF-κB, a transcription factor playing a crucial role in the development of gastro-intestinal inflammatory diseases [3,4]. NF-κB is deeply involved in the control of the transcription of several pro-inflammatory mediators, including IL-8, thus leading to the worsening of inflammatory conditions [5,6]. Moreover, activation of the NF-κB pathway in gastric epithelial cells is a hallmark of H. pylori-induced chronic inflammation and gastric carcinogenesis [7,8].

The search for new strategies able to interfere with these mechanisms by preventing a prolonged inflammation would greatly benefit a large number of subjects. In this respect, botanicals are widely consumed all over the world for health purposes, as different types of products, including herbal medicinal products, plant food supplements, and functional foods. Dietary guidelines recommend increased consumption of fruits and vegetables, including

http://dx.doi.org/10.1016/j.phrs.2016.07.028
1043-6618/© 2016 Elsevier Ltd. All rights reserved.
berries, as a good source of fibers and polyphenols [9]. In plants, one of the most important role of polyphenols is their involvement in the resistance mechanisms against environmental stresses [10,11]. Among these stresses, irrigation deficit can be exploited to increase polyphenol content in strawberries [12]. Emerging research provides substantial evidence to classify strawberries (Fragaria x ananassa Duch.) as a functional food with several preventive and therapeutic health benefits mainly versus chronic diseases [13,14]. Strawberries contain a variety of fat-soluble vitamins (vitamins A, E, K) and high amount of vitamin C, fibers and micronutrients [15]. In addition, strawberries are among the richest dietary sources of polyphenols, including anthocyanins mainly as pelargonidin and cyanidin glycosides [16]. Strawberries are also rich of condensed tannins (procyanidins) and hydrolysable tannins, especially ellagitannins such as agrimoniin [17–21].

Accumulating evidence from in vitro and in vivo studies ascribes to strawberry fruit several biological activities including antioxidrant [22], vasorelaxant [23], anti-inflammatory [23,24], hypolipidemic [25], and anti-diabetic [26,27] effects. Furthermore, inhibitory activity toward digestive enzymes after ingestion of berries, including strawberries, was reported [28]. Strawberries were shown to inhibit ethanol-induced gastritis in rats [22], and this effect was attributed to the presence of anthocyanins, whereas the contribution of tannins was not investigated. Recently, our group demonstrated that ellagitannin-enriched extracts from fruits of Rubus idaeus L. (raspberry) and Rubus fruticosus L. (blackberry) inhibit ethanol-induced gastritis in rats acting on the NF-κB pathway; the anti-inflammatory effect was ascribed, at least in part, to the presence of ellagitannins sanguin H-6 and lambertin C [29]. The aims of the present study were devoted to: i) the chemical characterization of strawberry tannins and evaluation of the biological activities involved in the attenuation of gastric inflammation; ii) the in vitro assessment of the impact of the gastric environment on the chemical and anti-inflammatory features of strawberry tannins; iii) the elucidation of the underlying molecular mechanisms.

Tannins-enriched extracts, obtained from strawberries (Fragaria x ananassa Duch.) and wild strawberries (Fragaria vesca L.), and pure tannins, were assayed on human gastric epithelial (AGS) cells stimulated with TNFα; this cell model was chosen because it shares close similarities with the gastric epithelium subject to H. pylori infection. The effect of the extracts and pure compounds on the NF-κB pathway (i.e. driven transcription and nuclear translocation) and IL-8 expression, secretion and promoter activity was investigated.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were HPLC or LC–MS grade and were purchased from Sigma Aldrich (Milan, Italy). Ellagic acid (purity >98%) was provided by Sigma Aldrich (Milan, Italy). Agrimoniin was isolated as described in Vrhossek et al. [30]; sanguin H-6 and casuaricin were isolated as described in [31] and [21], respectively. Other standards of polyphenols were purchased as described in [30] and [32].

2.2. Plant material and preparation of tannin enriched fraction

Strawberries (Fragaria X ananassa Duch., cv Darselect) and wild strawberries (Fragaria vesca L.) were grown in an experimental field in Vigalzano (Trento, Italy) under controlled conditions to reduce the impact of environmental and agronomic factors [21]. At harvest, fruits were healthy and the ripening stage was assessed as described [21]. Extraction of polyphenols from fruits was performed as reported by Gasperotti et al. [16]. Purification of the polyphenol fraction was carried out using an established method [31].

2.3. Cell culture

Human adenocarcinoma cells (AGS, CRL-1739, LGC Standard S.r.l., Milano, Italy) were grown at 37 °C in DMEM F12 (Gibco-Invitrogen) supplemented with 100 units penicillin/mL, 100 mg streptomycin/mL, 2 mM-glutamine and 10% heat-inactivated fetal calf serum (FCS) (Euroclone S.p.A, Pero, Italy) (complete medium), in a humidified atmosphere containing 5% CO2. Cells (passage number 20–30) were treated with the indicated concentrations of strawberry extracts or pure compounds or vehicle alone (<0.1% DMSO). The range of concentrations tested was based on the following considerations: i) enriched extracts are usually assayed at the maximal concentration of 10 μg/ml; (ii) selected concentrations are consistent with those achievable upon consumption of a regular serving of strawberries (100 g). Treatment with 20 μM epigallocatechin-3-gallate (EGCG) was used as reference compound.

2.4. NF-κB driven transcription and IL-8 promoter activity

To evaluate NF-κB driven transcription and IL-8 promoter activity, cells were plated in 24-well plates (30,000 cells/well). After 48 h cells were transiently transfected by the calcium-phosphate method with different reporter plasmids (NF-κB-LUC, 50 ng/well; IL-8-LUC, native or mutated, 100 ng/well); all plasmids contain luciferase gene under control of a specific promoter: NF-κB-LUC promoter possesses three κB responsive elements, while IL-8-LUC contains a fragment of the native promoter of the human IL-8, gene which is characterized by different responsive sequences for transcription factors such as activator protein 1 (AP-1), C/EBP- enhancer-binding protein-β (C/EBPβ), and NF-κB. The plasmid NF-κB-LUC was a gift of Dr. N. Marx (Department of Internal medicine- Cardiology, University of Ulm, Germany) while the native and mutated IL-8-LUC promoters were kindly provided by Dr. T. Shimohata (Department of Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan).

After 16 h, cells were placed in a medium deprived of FCS, and stimulated with TNFα at 10 ng/mL. Tannin-enriched extracts were tested at 0.05–5 μg/mL while individual compounds at 0.01–50 μM. After 6 h cells were lysed and luciferase assay was performed using BritelitePlus reagent (PerkinElmer Inc. Massachusetts, USA) according to manufacturer’s instructions; signal was read with VictorX3 (Perkin Elmer, Walthman MA, USA). Data were expressed considering 100% the luciferase activity related to the cytokine-induced NF-κB driven transcription or IL-8 promoter activity. The transcriptional activity of the IL-8-LUC mutated at the NF-κB binding site was evaluated in the same conditions described for the native promoter. These assays were performed in triplicate within each experiment.

2.5. NF-κB nuclear translocation

To assess the effects of the extracts and individual compounds on the NF-κB (p65) nuclear translocation, AGS cells were plated at the density of 1.5 × 10⁵ cells/mL in 100 mm plates. After 48 h, cells were treated for 1 h with the pro-inflammatory mediators and the extracts/compounds under study. Nuclear extracts were prepared using Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA) and stored at −80 °C until assayed. The same amount of total nuclear proteins (10 μg/well), measured by the method of Bradford (Bio-Rad), was used to assess the NF-κB nuclear translocation using the NF-κB (p65) transcrip-
tion factor assay kit (Cayman) followed by spectroscopy at 450 nm, 0.1 s (VictorX3, Perkin Elmer, Walthman MA, USA). Data were expressed considering 100% the absorbance related to the cytokine-induced NF-κB nuclear translocation. These assays were performed in duplicate within each experiment.

2.6. Measurement of IL-8 levels

For measurement of IL-8 secretion, AGS cells were grown in 24-well plates (30,000 cells/well) for 48 h; then, cells were treated with pro-inflammatory stimuli (TNFα at 10 ng/ml) and extracts/compound under study. After 6 h treatment the medium was removed and stored at −20 °C until the assay.

IL-8 was quantified by an enzyme-linked immunosorbent assay (ELISA) Kit (Peprotech, Rocky Hill, NJ, USA) as previously described [33]. The IL-8 secretion reached the maximum at 6 h and this time was selected for the experiments to test the effect of the extracts (0.1–5 μg/ml) and individual compounds (0.05–50 μM). These assays were performed in duplicate within each experiment.

2.7. In vitro gastric digestion of strawberry extract

To evaluate the effect of in vitro digestion on the activity of the strawberry extract we used the protocol previously reported [33]. Strawberry extract (200 mg) was mixed with 2 mL of saliva and gastric juice solution and incubated for 5 min under constant shaking at 37 °C. After this first step 4 mL of gastric juice were added and incubated for 2 h at 37 °C in constant agitation. The efficacy of the conditions used to simulate gastric digestion was confirmed in a previous study [33]. The solution obtained was dried and frozen at −80 °C until analysis. The digested extract obtained was referred to 200 mg of the original extract and tested on different biological activities comparing the effect to non-treated control adjusted with a mixture of saliva and gastric juice.

2.8. Quantification of tannin-enriched extracts and in vitro digested extracts

The quantification of the ellagitannins and the other polyphenols were performed using a Waters Aquity system coupled with a triple quadrupole (TQ) mass spectrometer Waters UHPLC Xevo TQ (Millford, Massachusetts, USA) applying the method for the quantification of 130 polyphenols and 23 polyphenol microbial metabolites adapted from [16,30,32].

In addition, proanthocyanidins present in tannin-enriched extracts and in vitro digested extracts were quantified by the vanillin-HCl method (or vanillin index) following the protocol described by Rigo et al. [34]. Proanthocyanidins were expressed as equivalent of (+)-catechin.

2.9. Cytotoxicity

The citotoxicity of tannins-enriched extracts and single compounds was evaluated by the 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [33].

2.10. Statistical analysis

All data are expressed as mean ± s.d. of at least four experiments. The number of experiments for each assay is specified in the figure legends; data were analyzed by unpaired one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). p < 0.05 was considered statistically significant. IC_{50}s were calculated using GraphPad Prism 5.00 software.

3. Results

3.1. Characterization of tannin-enriched extracts from strawberries

The detailed chemical characterization of the tannin-enriched strawberry extracts before and after in vitro digestion is reported in Table 1. A total of 41 different polyphenols were detected in both undigested extracts. Agrimonin and casuaricin were the main ellagitannins occurring in strawberry extracts, in line with published data referring to strawberries [21]. The second most abundant class of polyphenols was procyanidins (procyanidin B1–B4) and oligomeric and polymeric proanthocyanidins. Proanthocyanidins are one of the most representative classes of polyphenols in strawberries [16] and they were retained efficiently during the purification of the extracts similarly to the ellagitannins due to their oligomeric structure. Other classes of polyphenols, such as flavonones, flavanones, flavonols, dihydrochalcones were present in lower amounts, listed in Table 1. Anthocyanins instead were not present because they were removed during the purification of the tannin-enriched extract.

3.2. Tannin-enriched extracts from strawberries inhibit IL-8 secretion in TNFα-treated AGS cells

First of all, we investigated the ability of tannin-enriched extracts from strawberries and wild strawberries to affect NF-κB signaling in AGS cells treated with TNFα. As shown in Fig. 1, both extracts inhibited NF-κB driven transcription (panel A and B) and p65 translocation (panel C and D) in a concentration-dependent manner.

To test the effect of strawberry extracts on NF-κB driven transcription, AGS cells were transiently transfected with the NF-κB-LUC plasmid (50 ng/well) by the calcium-phosphate method. After 16 h, cells were treated with TNFα (10 ng/ml) in the presence of increasing concentrations of tannin-enriched extracts from strawberries (panel A, n = 5) or wild strawberry (panel B, n = 5) for 6 h. To test the effect of strawberry extracts on NF-κB translocation AGS cells were treated with TNFα (10 ng/ml) in the presence of increasing concentrations of tannin-enriched extracts from strawberries (panel C, n = 6) or wild strawberries (panel D, n = 6) for 6 h. The amount of p65 in the nuclear fraction was measured by ELISA and normalized by protein content. The graphs show the means ± s.d. of the indicated number (n) of experiments. The treatment with the reference compound (20 μM EGCG) yielded the expected inhibition of the tested parameters: >85% inhibition of NF-κB driven transcription and >90% inhibition of p65 translocation. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus TNFα alone. The schemes in panel A and B depict the structure of the NF-κB-LUC plasmid containing three copies of the E-selectin κB site.

This observation is consistent with previous results showing that tannin-enriched extracts from other origins, such as Rubus berries, dampen NF-κB signaling in TNFα-stimulated AGS cells [29]. Of note, the strawberry extract appeared slightly more active in comparison to that from wild strawberries. In fact, the IC_{50} related to NF-κB reporter activity were 0.23 μg/ml for strawberries and 0.42 μg/ml for wild strawberries (Table 2); while the IC_{50} related to p65 translocation were 0.30 μg/ml for strawberries and 0.79 μg/ml for wild strawberries (Table 2).
Table 1. Chemical composition of tannin-enriched extracts from strawberries.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tannins enriched extract</th>
<th>in vitro digested extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strawberry</td>
<td>wild strawberry</td>
</tr>
<tr>
<td>methyl gallate</td>
<td>µg/µg</td>
<td>µg/µg</td>
</tr>
<tr>
<td>fraxin</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>trans-caftaric acid</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>trans-piceide</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>cis-piceide</td>
<td>6.0</td>
<td>4.8</td>
</tr>
<tr>
<td>phlorizin</td>
<td>28.6</td>
<td>1.0</td>
</tr>
<tr>
<td>trans-piceide</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>trilobatin</td>
<td>366.2</td>
<td>31.8</td>
</tr>
<tr>
<td>luteolin</td>
<td>39.4</td>
<td>2.5</td>
</tr>
<tr>
<td>luteolin-7-O-glucoside</td>
<td>64.8</td>
<td>118.4</td>
</tr>
<tr>
<td>naringenin</td>
<td>8.8</td>
<td>1.3</td>
</tr>
<tr>
<td>catechin</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>epicatechin</td>
<td>34.6</td>
<td>653.0</td>
</tr>
<tr>
<td>procyanidin B1</td>
<td>15630.6</td>
<td>15553.1</td>
</tr>
<tr>
<td>procyanidin B2 + B4 (as eq. Procyanidin B2)</td>
<td>1660.8</td>
<td>2510.5</td>
</tr>
<tr>
<td>procyanidin B3 (as eq. Procyanidin B1)</td>
<td>27270.7</td>
<td>18310.6</td>
</tr>
<tr>
<td>kaempferol</td>
<td>112.7</td>
<td>12.5</td>
</tr>
<tr>
<td>quercetin</td>
<td>24.2</td>
<td>6.8</td>
</tr>
<tr>
<td>taxifolin</td>
<td>7.3</td>
<td>10673.2</td>
</tr>
<tr>
<td>kaempferol-3-glucoside</td>
<td>1164.9</td>
<td>494.6</td>
</tr>
<tr>
<td>kaempferol-3-rutinoside</td>
<td>99.1</td>
<td>2.4</td>
</tr>
<tr>
<td>dihydrokaempferol</td>
<td>78.8</td>
<td>49.9</td>
</tr>
<tr>
<td>quercetin-3-glucuronide</td>
<td>1815.4</td>
<td>175.0</td>
</tr>
<tr>
<td>kaempferol-3-glucuronide</td>
<td>1197.8</td>
<td>19.9</td>
</tr>
<tr>
<td>4-hydroxybenzoic acid</td>
<td>229.1</td>
<td>156.1</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>11.1</td>
<td>0.2</td>
</tr>
<tr>
<td>gallic acid</td>
<td>2.1</td>
<td>6.8</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>2.4</td>
<td>0.1</td>
</tr>
<tr>
<td>trans-ferulic acid</td>
<td>1.1</td>
<td>0.0</td>
</tr>
<tr>
<td>urolithin A</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>ellagic acid</td>
<td>1675.9</td>
<td>2378.5</td>
</tr>
<tr>
<td>pyrocatechol</td>
<td>116.6</td>
<td>106.9</td>
</tr>
<tr>
<td>quercetin-3-glucoside + quercetin-3-galactoside (as eq. quercetin-3-glucoside)</td>
<td>148.7</td>
<td>798.3</td>
</tr>
<tr>
<td>isorhamnetin-3-glucoside</td>
<td>15.4</td>
<td>317.8</td>
</tr>
<tr>
<td>methyl ellagic acid rhamnoside</td>
<td>19.9</td>
<td>7697.2</td>
</tr>
<tr>
<td>agrimonin</td>
<td>42903.8</td>
<td>52297.8</td>
</tr>
<tr>
<td>casuaricin</td>
<td>46471.3</td>
<td>23169.2</td>
</tr>
<tr>
<td>sanguin H6</td>
<td>2241.0</td>
<td>2069.6</td>
</tr>
<tr>
<td>Total polyphenols (MS/MS analysis)</td>
<td>162988.4</td>
<td>154868.1</td>
</tr>
<tr>
<td>proanthocyanidins</td>
<td>485576.0</td>
<td>360983.2</td>
</tr>
<tr>
<td>Total polyphenols (MS/MS analysis and proanthocyanidins)</td>
<td>648564.4</td>
<td>515851.3</td>
</tr>
</tbody>
</table>

Fig. 1. Tannin-enriched extracts from strawberries and wild strawberries inhibit NF-κB signaling in TNFα-treated AGS cells.
Table 2
IG50 of tannin-enriched extracts and strawberry tannins for the tested biological activities.

<table>
<thead>
<tr>
<th>Biological process</th>
<th>NF-κB signaling pathway</th>
<th>IL-8 expression and secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p65 translocation</td>
<td>NF-κB driven transcription</td>
</tr>
<tr>
<td>Biological assay</td>
<td>0.30 µg/ml</td>
<td>0.23 µg/ml</td>
</tr>
<tr>
<td>Strawberry</td>
<td>0.79 µg/ml</td>
<td>0.42 µg/ml</td>
</tr>
<tr>
<td>Ferronin</td>
<td>1.71 µg/ml</td>
<td>1.07 µg/ml</td>
</tr>
<tr>
<td>Agrimomnin</td>
<td>0.81 µM</td>
<td>0.50 µM</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>1.68 µM</td>
<td>0.44 µM</td>
</tr>
<tr>
<td>Sanguin H-6</td>
<td>0.87 µM</td>
<td>1.5 µM</td>
</tr>
<tr>
<td>Casuarin</td>
<td>0.33 µM</td>
<td>0.29 µM</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>inactiveb</td>
<td>inactiveb</td>
</tr>
</tbody>
</table>

Results are the mean of at least four experiments.

a Data refer to reference [29].
b Maximum concentration tested 50 µM.

When we examined the effects on IL-8 secretion and promoter activity in TNFα-treated cells, we found a concentration-dependent inhibition following co-treatment with tannin-enriched extracts from both strawberries (Fig. 2, panel A and C) and wild strawberries (panel B and D).

To test the effect of strawberry extracts on IL-8 secretion AGS cells were treated with TNFα (10 ng/ml) in the presence of increasing concentrations of tannin-enriched extracts from strawberries (panel A, n = 6) or wild strawberries (panel B, n = 6) for 6 h. The amount of released IL-8 was measured by ELISA assay. To test the effect of strawberry extracts on IL-8 transcription, AGS cells were transiently transfected with a plasmid carrying the luciferase gene under the control of a fragment of the IL-8 promoter containing several responsive sequences (the structure is depicted in the scheme, see text for details) (100 ng/well) by the calcium-phosphate method. After 16 h, cells were treated with TNFα (10 ng/ml) in the presence of increasing concentrations of tannin-enriched extracts from strawberries (panel C, n = 5) or wild strawberries (panel D, n = 5) for 6 h. The graphs show the means ± s.d. of the indicated number (n) of experiments. The treatment with the reference compound (20 µM EGCG) yielded the expected inhibition of the tested parameters: >70% inhibition of IL-8 secretion, and >80% inhibition of IL-8 promoter activity. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα alone.

Notably, the IL-8 promoter, carrying a mutation at the NF-κB binding site, lost responsiveness to TNFα and, consequently, to strawberry extract (Supplementary material Fig. S1). These results are in agreement with the data reported in Fig. 1 and indicate that inhibition of IL-8 promoter activity operates through impairment of NF-κB signaling. We verified that the treatment with TNFα does not activate Activator Protein-(AP)-1 by assaysing the transcriptional activity of a reporter plasmid carrying the corresponding binding site upstream the luciferase gene (data not shown).

3.3. Most of the biological properties of a tannin-enriched extract from strawberries are preserved after in vitro simulated gastric digestion

To test the chemical endurance of the tannin-enriched extract from strawberries to the harsh conditions of the stomach environment, we simulated in vitro the gastric digestion as described in the Materials and methods section.

The chemical analysis of the material subject to simulated gastric digestion (Table 1) revealed that among ellagitannins, agrimoniin was affected the most (26% loss with respect to 8% loss of casuaricin). Also the procyanidin fraction underwent chemical modification, in fact the content of procyanidin B1 increased by 26% while that of procyanidin B3 decreased by 35%. Almost negligible degradation of ellagitannins to urolithins was observed since only minor amounts of urolithin A (7.9 µg/g) were formed, starting from about 90 mg/g of agrimoniin and casuaricin.
The relative stability of casuaricin, agrimonin and sanguin H-6 to hydrolysis can be inferred also from the modest release of gallic acid and ellagic acid following in vitro digestion (25.9 μg/g and 169.7 μg/g, respectively).

As shown in Fig. 3 the tannin-enriched extract subject to simulated digestion retained most of the biological activities described above. While the inhibitory effect on IL-8 secretion was unchanged (Fig. 3 panel A vs. Fig. 2 panel A), a slight loss of activity was observed in the case of TNFα-induced activation of NF-κB signaling. In fact, completed prevention of stimulated p65 translocation (panel B), NF-κB driven transcription (panel A), and IL-8 promoter activity (panel D) was achieved at higher concentrations (about 5 μg/ml) with respect to the native extract (about 2.5 μg/ml) (Fig. 1 panel C and A, Fig. 2 panel C, respectively).

AGS cells were transiently transfected with the NF-κB-LUC plasmid (panel A, n = 5) or the wild type IL-8 promoter (panel D, n = 5) and then treated as described in the legend to Figs. 1 and 2. Similarly, the amount of p65 in the nuclear fraction (panel B, n = 6) and the amount of IL-8 released into the medium (panel C, n = 6) was measured as indicated in the legend to Figs. 1 and 2. The graphs show the means ± s.d. of the indicated number (n) of experiments. The treatment with the reference compound (20 μM EGCG) yielded the expected inhibition of the tested parameters: >85% inhibition of NF-κB driven transcription; >90% inhibition of p65 translocation; >70% inhibition of IL-8 secretion; >80% inhibition of IL-8 promoter activity. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα alone.

Our results confirmed that one of the molecular targets of ellagitannins is the NF-κB pathway, as demonstrated by the inhibitory effects observed on promoter activity of the NF-κB reporter system (Figs. 4 A and 5 A), p65 translocation (Figs. 4 B and 5 B), and the lack of effect on the IL-8 mutated promoter lacking the κB site (data not shown). Most of the biological activities displayed by agrimonin and casuaricin highly resembled those reported for structurally similar ellagitannins, such as sanguin H-6 and lambertianin C, in the same experimental setting [29]. To fully characterize sanguin H-6, an isomer of agrimonin, we assayed its effect on IL-8 promoter activity (Supplementary material Fig. S2), confirming previously published and present data.

In our extracts free ellagic acid was present at biologically significant concentrations, 1.7 and 2.7 mg/g of strawberry and wild strawberry extract, respectively (Table 1). Hence, we inferred that ellagic acid might contribute to the biological activities exhibited by the tannin-enriched extracts shown above. Moreover, from the structure-activity point of view, the moiety of ellagic acid is a common feature of many ellagitannins found in strawberries and other natural sources. For these reasons we decided to investigate the biological activities of ellagic acid in TNFα-treated AGS cells and Fig. 6 summarizes our findings. Similarly to agrimonin and casuaricin, ellagic acid (0.1–5 μM) counteracted the activation of IL-8 secretion elicited by TNFα in an NF-κB-dependent manner.

AGS cells were transiently transfected with the NF-κB-LUC plasmid (panel A, n = 5) or the wild type IL-8 promoter (panel D, n = 6) and then treated as described in the legend to Figs. 1 and 2. Similarly, the amount of p65 in the nuclear fraction (panel B, n = 6) and the amount of IL-8 released into the medium (panel C, n = 4) was measured as indicated in the legend to Figs. 1 and 2. The graphs show the means ± s.d. of the indicated number (n) of experiments. The treatment with the reference compound (20 μM EGCG) yielded the expected inhibition of the tested parameters: >85% inhibition of NF-κB driven transcription; >90% inhibition of p65 translocation; >70% inhibition of IL-8 secretion; >80% inhibition of IL-8 promoter activity. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα alone.

3.4. Ellagitannins prevent IL-8 secretion by TNFα-treated AGS cells through inhibition of NF-κB signaling

As shown in Table 1 agrimonin and casuaricin were present at comparable concentration in strawberry extract. Therefore, we examined the effects of the pure compounds on NF-κB signaling and IL-8 expression and release. As reported in Fig. 4, agrimonin (0.1–2.5 μM) exhibited biological properties similar to those of strawberry extracts.

AGS cells were transiently transfected with the NF-κB-LUC plasmid (panel A, n = 5) or the wild type IL-8 promoter (panel D, n = 4) and then treated as described in the legend to Figs. 1 and 2. Similarly, the amount of p65 in the nuclear fraction (B, n = 6) and the amount of IL-8 released into the medium (panel C, n = 5) was measured as indicated in the legend to Figs. 1 and 2. The graphs show the means ± s.d. of the indicated number (n) of experiments. The treatment with the reference compound (20 μM EGCG) yielded the expected inhibition of the tested parameters: >85% inhibition of NF-κB driven transcription; >90% inhibition of p65 translocation; >70% inhibition of IL-8 secretion; >80% inhibition of IL-8 promoter activity. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα alone.

Similar results were obtained in AGS cells co-treated with TNFα and casuaricin (Fig. 5) (0.01–5 μM).

AGS cells were transiently transfected with the NF-κB-LUC plasmid (panel A, n = 5) or the wild type IL-8 promoter (panel D, n = 5) and then treated as described in the legend to Figs. 1 and 2. Similarly, the amount of p65 in the nuclear fraction (panel B, n = 6) and the amount of IL-8 released into the medium (panel C, n = 6) was measured as indicated in the legend to Figs. 1 and 2. The graphs show the means ± s.d. of the indicated number (n) of experiments. The treatment with the reference compound (20 μM EGCG) yielded the expected inhibition of the tested parameters: >85% inhibition of NF-κB driven transcription; >90% inhibition of p65 translocation; >70% inhibition of IL-8 secretion; >80% inhibition of IL-8 promoter activity. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα alone.

3.5. Strawberry tannins inhibit IL-8 secretion through different mechanisms

As reported in Table 1 the strawberry extracts contained significant amounts of condensed tannins, such as procyanidin B1 and B3; therefore, we decided to evaluate the biological activities of procyanidin B1 as representative member of this class of compounds. We found that 1 μM procyanidin B1 inhibited TNFα-induced IL-8 secretion by 44%, in an NF-κB-independent manner since no effect on p65 translocation and NF-κB-driven transcription was observed at concentration as high as 50 μM (Supplementary material Fig. S3, Table 2).

To gain mechanistic insights and some hints on structure-related biological activities we compared the IC50 of pure strawberry tannins relative to the tested biological activities (Table 2). Prevention of TNFα-induced secretion of IL-8 by agrimonin occurred at concentrations much lower than those required to inhibit NF-κB driven transcription (0.09 μM vs 0.50 μM). A similar
Fig. 3. In vitro simulated gastric digestion barely attenuates the biological activities of strawberry tannins.

Fig. 4. Agrimonin inhibits IL-8 promoter activity and secretion in an NF-κB-dependent manner in TNFα-treated AGS cells.

trend applies to sanguin H-6; on the other hand, ellagic acid is more potent at inhibiting the promoter activity of the NF-κB reporter (0.44 μM) than IL-8 secretion (2.56 μM). Casuarictin prevented IL-8 secretion at the same concentrations required to dampen p65 translocation and NF-κB driven transcription, indicating that this ellagitannin affects IL-8 solely through inhibition of the NF-κB pathway. It is worth noting that the two isomers agrimonin and sanguin H-6 inhibited IL-8 secretion by TNFα-treated cells with considerably different potencies (IC50s of agrimonin and sanguin H6: 0.09 μM and 0.58 μM, respectively).

Altogether, these results suggest that in our experimental setting: i) casuarictin may be a pure NF-κB inhibitor while agrimonin and sanguin H-6 most likely inhibit IL-8 secretion also acting on other biological targets; ii) the ellagic acid moiety contributes to the biological activity of ellagitannins but other chemical and stereo-chemical features impact their ability to interact with biological targets; iii) the representative condensed tannin procyanidin B1, prevents the TNFα-induced effects without interfering with the NF-κB pathway.

4. Discussion

Population-based studies and research in preclinical models provide substantial evidence to classify strawberries as a functional food with preventive and therapeutic benefits versus common diseases, most of which are characterized by chronic inflammation [13]. The gastro-intestinal tract, especially the stomach, is the first site of action of functional foods; therefore, the ability to atten-
ulate inflammatory pathways and chemical stability in the gastric environment are required features of stomach-targeting bioactive compounds. In our study we add experimental support that strawberry constituents, most of which are polyphenols, meet the over mentioned requirements.

From the mechanistic point of view, strawberry polyphenols may exert their protective effects by reducing the oxidative stress [22] and/or by attenuating inflammatory pathways, e.g. AP-1 and NF-κB activities, and MAPK signaling [35]. Indeed, the antioxidant actions of the anthocyanin fraction were proposed as the mechanism underlying the anti-ulcerative properties of a hydroalcoholic strawberry extract [22]. However, strawberry polyphenols may exert their beneficial effects acting by different mechanisms beyond their antioxidant capacity, as recently reviewed [36,37]. Given the current knowledge on the chemical diversity of strawberry polyphenols [16,21], the contribution to the biological activities of other compounds, i.e. ellagitannins, should also be considered and investigated.

Our results in a cell model of gastric inflammation clearly show that strawberry tannins significantly attenuate the release of IL-8, a key mediator in gastric diseases, at concentrations lower than 1 μg/mL. Considering the average volume of gastric content in adult individuals during a meal (0.6 L), this concentration can be easily achieved upon consumption of a regular serving of fresh strawberries (100 g) [16]. Coherently with their chemical composition, tannin-enriched extracts from both *Fragaria* species inhibit IL-8 secretion by TNFα-treated cells in a similar fashion. Moreover, the biological activities of strawberry tannins are maintained upon simulated digestion, consistently with the slight changes in the chemical profile. These observations are in line with the previ-
ously reported stability of ellagitannins derived from other sources in acidic environments [38,39] and the presence of unmodified procyanidins in the gut [40]. Altogether, our results support the concept that regular consumption of strawberries may contribute to protect against gastric inflammatory diseases.

In the present study we demonstrate that the anti-inflammatory activity of strawberry tannins is strongly, although not exclusively, related to inhibition of the NF-κB pathway, a central player in inflammatory diseases, including gastritis [41]. In the model system used in our study, the activity of the AP-1 complex is unaffected by the inflammatory stimulus and by strawberry tannins, while modulation of MAPKs cannot be excluded. We used pure compounds to dissect the contribution of the major components of strawberry tannins to the anti-inflammatory activities focusing on: agrimonin and casuracin, as the main ellagitannins found in these berries, ellagic acid, as a common chemical determinant of ellagitannins, and procyanidin B1, as representative of condensed tannins.

Our results indicate that agrimonin, similarly to its isomer sanguin H-6, dampens IL-8 secretion by inhibiting the NF-κB pathway and by acting on other targets, except AP-1. These results are consistent with previous reports on the anti-inflammatory and immunomodulatory use of plants containing agrimonin, such as Potentilla spp. [42], and Agrimonia spp. [43,44], and the ability of pure agrimonin to prevent LPS-induced secretion of IL-8 by human neutrophils [45]. Here we show for the first time that pure casuaricin exhibits anti-inflammatory properties with high selectivity for the NF-κB pathway, at least in TNFα-treated AGS cells. The effect of ellagic acid in the stomach has been previously reported (for a review on this topic, please refer to [46]); the present results demonstrate that ellagic acid inhibits the NF-κB pathway and IL-8 secretion in epithelial gastric cells at concentrations as low as 1–2 μM that could be easily reached upon moderate consumption of strawberries. Being the ellagic acid moiety present in all ellagitannins, it is conceivable that it may contribute to the biological activities of these compounds.

Given the abundance of condensed tannins in the extracts used in the present study, we cannot exclude their contribution to the anti-inflammatory activity, in addition to ellagitannins. Procyanidin B1, which was selected as representative condensed tannin, is widely present in edible plants and likely contributes to their anti-inflammatory activity at the gastro-intestinal level [47]. The mechanisms by which procyanidin B1 inhibits LPS-induced inflammation mainly involve attenuation of NF-κB signaling as previously reported [48]. Here we show that procyanidin B1 can attenuate IL-8 secretion without affecting the NF-κB pathway in AGS cells subject to TNFα treatment.

5. Conclusions

In conclusion, our study demonstrates that strawberry tannins, either as enriched extract or as pure compounds, act on gastric epithelial cells by inhibiting the inflammatory response to TNFα through NF-κB-dependent and independent mechanisms. While procyanidins are widely present in natural sources and their biological activities have been well documented, ellagitannins can be found in some foods only and represent an emerging class of phytochemicals. According to available data, agrimonin and casuracin could be considered the most consumed ellagitannins in the world because of the large presence of strawberries in the human diet [30]. The anti-inflammatory action at the stomach level, coupled with the previously reported anti-H. pylori activity [49], makes strawberry ellagitannins exploitable as preventive or co-adjuvant agents in gastric diseases.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

Dr. T. Shimohata and Prof. A. Takahashi, Departments of Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan, provided the plasoids carrying the native and mutated IL-8 promoters. Domenico Masuero is acknowledged for excellent analytical support.

This research was supported by ADP and the GMF International PhD program funded by the Autonomous Province of Trento, Italy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.07.028.

References


