Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2014, **5**, 2981

Pitanga (*Eugenia uniflora* L.) fruit juice and two major constituents thereof exhibit anti-inflammatory properties in human gingival and oral gum epithelial cells

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Received 12th June 2014,
Accepted 3rd September 2014

DOI: 10.1039/c4fo00509k

www.rsc.org/foodfunction

Pitanga, *Eugenia uniflora* L., is a tropical fruit, which may be consumed as juice. While beneficial health effects of *Eugenia uniflora* L. leaf extracts have extensively been studied, limited data are available on an anti-inflammatory potential of pitanga juice. The aim of the presented study was to investigate anti-inflammatory properties of pitanga juice with regards to a prevention of inflammation-related periodontal diseases. For this purpose, six healthy volunteers swirled pitanga juice, containing 35% pitanga pulp, for 10 min. Thereafter, oral gum epithelial cells were harvested using a sterile brush and stimulated with lipopolysaccharides from *Porphyromonas gingivalis* (*PG*-LPS) for 6 h. Furthermore, human gingival fibroblasts (HGF-1) were used to elucidate the anti-inflammatory potential of pitanga juice constituents, cyanidin-3-glucoside and oxidoselina-1,3,7(11)-trien-8-one, in juice representative concentrations of 119 μg ml^{-1} and 30 μg ml^{-1}, respectively. For the first time, an anti-inflammatory impact of pitanga juice on gingival epithelial cells was shown by means of an attenuation of IL-8 release by 55 ± 8.2% and 52 ± 11% in non-stimulated and *PG*-LPS-stimulated cells, respectively. In addition, both cyanidin-3-glucoside and oxidoselina-1,3,7(11)-trien-8-one reduced the LPS-stimulated *CXCL8* mRNA expression by 50 ± 15% and 37 ± 18% and IL-8 release by 52 ± 9.9% and 45 ± 3.7% in HGF-1 cells, when concomitantly incubated with 10 μg ml^{-1} *PG*-LPS for 6 h, revealing an anti-inflammatory potential of the volatile compound oxidoselina-1,3,7(11)-trien-8-one for the first time.

Introduction

The pitanga plant, *Eugenia uniflora* L., belongs to the Myrtaceae family and is native to tropical and subtropical regions. The tree produces a fruit, called pitanga or Brazilian cherry, which is commonly used to produce juice, pulp, ice-cream, soft drinks, jams, liqueurs and wines.[1] Over the last three decades, the pharmacological potential of *Eugenia uniflora* L. leaf extracts has been extensively investigated.[2–5] Leaf extracts of the pitanga plant have been used in traditional South American medicine[5] to treat digestive disorders or hypertension, and possess anti-oxidant and anti-inflammatory activities.[3] However, data on beneficial health effects of pitanga fruit and products thereof, like pitanga juice, are sparse.

It is well known that the pitanga fruit contains a variety of carotenoids[1] and anthocyanins.[3,6] In a previous study, our group quantified two major compounds of the volatile and non-volatile fraction of pitanga fruit pulp, oxidoselina-1,3,7(11)-trien-8-one and the anthocyanin cyanidin-3-glucoside, respectively.[7] Anthocyanin-rich fruits exploit anti-inflammatory properties *in vitro*[8] and *in vivo*,[9] inhibiting the translocation of NF-κB into the nucleus in macrophages in concentrations ≥5 μg ml^{-1}.[10]

Inflammation is related to various diseases including gingivitis and periodontitis, inflammatory disorders of oral gum tissue which, in the most severe state, may lead to tooth loss.[11,12] A cause of inflammatory disease in the oral cavity is insufficient oral hygiene accompanied by an accumulation of bacterial plaque. Gram-negative oral bacteria, like *Porphyromonas gingivalis* (*PG*), present lipopolysaccharides (LPS) on their surface, evoking an immune response of the host.[13–15] Human gingiva contains fibroblasts to fight pathogens by toll-like

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receptor-mediated release of cytokines and chemokines.[13,16] A model system of human gingival fibroblasts (HGF-1) has been established in our group to identify anti-inflammatory properties of food compounds in the oral cavity.[17,18] Stimulation of HGF-1 cells with 10 μg ml^{-1} *PG*-LPS for 6 h led to the release of the pro-inflammatory chemokine interleukin 8 (IL-8) into cell culture supernatant.[17] Concomitant treatment with volatile compounds, like 1,8-cineole, decreased the amount of released IL-8, indicating an anti-inflammatory effect of this volatile compound.[18]

In the here presented study, we aimed to identify anti-inflammatory properties of cyanidin-3-glucoside and oxidoselina-1,3,7(11)-trien-8-one in concentrations found in pitanga juice on human oral tissue cells stimulated with *PG*-LPS *ex vivo* and *in vitro*.

Material and methods

Chemicals

Cyanidin-3-glucoside was obtained from Polyphenols Laboratories AS (Sandnes, Norway). The bead kit for IL-8 analysis was purchased from eBioscience (Vienna, Austria), while chemicals were bought from Sigma-Aldrich (Vienna, Austria), unless indicated otherwise.

Preparation of pitanga juice

Original Brazilian pitanga pulp (*Eugenia uniflora* L.) from a farm located in the city of Gandu, Bahia, Brazil was sent to Vienna, Austria on dry-ice to perform cell culture analyses. Recently, we published the quantification of the volatile compound, oxidoselina-1,3,7(11)-trien-8-one, and the non-volatile compound, cyanidin-3-glucoside in pitanga pulp.[7] Generally, the pulp is processed to juice by addition of sugar and water before consumption. Pitanga juice was prepared according to the Brazilian law, using 35% pitanga pulp and 7.1% glucose in tap water, resulting in a pH of 3.26 ± 0.01. Based on mean concentrations of cyanidin-3-glucoside (340 μg ml^{-1}) and oxidoselina-1,3,7(11)-trien-8-one (85 μg ml^{-1}) present in the pulp,[7] a calculated concentration of 119 μg ml^{-1} cyanidin-3-glucoside and 30 μg ml^{-1} of oxidoselina-1,3,7(11)-trien-8-one was used in the present study addressing the anti-inflammatory potential of pitanga juice.

Exposure of human gingival cells to pitanga juice and *PG*-LPS stimulation *ex vivo*

Experiments were performed with six healthy volunteers (age: 22 to 33 years, all female), who were neither allowed to have breakfast nor to brush their teeth in the morning before the intervention. Volunteers agreed to participation by signing a written consent form.

To exclude effects of drinking and swirling a drink, a control sample containing solely 7.1% glucose and water was prepared. Volunteers were asked to swirl 100 ml of pitanga juice or control drink in their mouths for a total of 10 min. The total juice volume was split into 3 aliquots, two aliquots of 30 ml for 3 min each and one aliquot of 40 ml for 4 min. After the swirling and waiting for 5 min, the cells from the outer epithelial of the gums on both sides of the mouth were collected with a sterile brush (Cytobrush®, Mpoe, Klagenfurt, Austria) and placed in 5 ml RPMI medium supplemented with 10% FBS, 8 mM glutamine, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. Cells were shaken for 10 minutes at room temperature, counted using trypan blue staining with a hemocytometer, split in half and centrifuged at 400*g* for 5 min at 20 °C. The cells were then resuspended in medium with or without 10 μg ml^{-1} *PG*-LPS (InvivoGen, San Diego, CA, USA) to a concentration of 150 000 viable cells ml^{-1} and incubated at 37 °C and 5% CO_2 for 6 hours. After the six hour incubation, the supernatant was removed and stored for analysis of IL-8 release at −80 °C. To determine the IL-8 release, a commercial kit (Human Basic FlowCytomix, eBioscience) was used according to the manufacture's protocol. Beads were incubated with 25 μl of sample in the dark for 2 h, and fluorescence was detected with a guava easyCyte™ HT flow cytometer (Merck-Millipore, Vienna, Austria).

HGF-1 cell culture, *PG*-LPS and compound incubation

HGF-1 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 20% fetal bovine serum, 8 mM glutamine, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin at 37 °C and 5% CO_2 in a humidified incubator.

For each experiment, HGF-1 cells were incubated at 37 °C and 5% CO_2 with 1% DMSO as a solvent control or 10 μg ml^{-1} *PG*-LPS plus 1% DMSO. Gene expression of *CXCL8* in HGF-1 cells after stimulation with 10 μg ml^{-1} *PG*-LPS was measured at five different time points, 0.5, 1, 3, 6 and 24 hours by RT-qPCR. Compound incubation was done in combination with 10 μg ml^{-1} *PG*-LPS. The single compounds, 119 μg ml^{-1} cyanidin-3-glucoside and 30 μg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one, were dissolved in DMSO, while pitanga juice was prepared as described above using water and incubation media (1 : 1, pH 4.04 ± 0.01). Treatments were performed either for 10 min with compound, followed by 6 h LPS stimulation (pre-incubation) or as a concomitant incubation of compound and *PG*-LPS for 6 h (co-incubation).

Toxicity of pitanga juice and two juice constituents in HGF-1 cells

Cytotoxic effects of pitanga juice on the viability of HGF-1 cells were determined after incubation of the cells with pitanga juice or the individual pitanga constituents. Therefore, HGF-1 cells were treated with pitanga juice, 119 μg ml^{-1} cyanidin-3-glucoside and 30 μg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one for 10 min, followed by 6 h stimulation with 10 μg ml^{-1} *PG*-LPS or concomitantly incubated with the pitanga compound and 10 μg ml^{-1} *PG*-LPS for 6 h. The cell viability after the treatments was normalized to the viability of solvent control cells (T/C in %). Cytotoxicity was determined by ViaCount (Merck-Millipore) assay using flow cytometry. For this analysis, cells

were seeded into a 24-well plate containing 50 000 HGF-1 cells per well, maintained in medium and incubated at 37 °C, 5% CO_2 for two days. After this time, the medium was removed and 300 µl of medium containing the substances were added for 10 min, then removed; and the cells were treated with 10 µg ml^{-1} *PG*-LPS for 6 h. The experiments were also conducted in the presence of 10 µg ml^{-1} *PG*-LPS plus compound for 6 h. Thereafter, the medium was removed. Cells were washed with 500 µl of phosphate buffered saline and detached with trypsin. Then, cells were pelleted by centrifugation at 4 °C and 400*g* for 7 min and resuspended in 180 µl ViaCount reagent (Merck-Millipore). The measurement using the flow cytometer (guava easyCyte™ HT) was performed according to the manufacture's protocol. The viability readouts of the treated cells were compared to the solvent control.

Gene expression analysis in HGF-1 cells

The RNA of treated cells was isolated using the SV Total RNA Isolation System (Promega, Mannheim, Germany). Thereafter, RNA samples were quantified at 260 nm using a Nanoquant plate (Tecan, Austria). The RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Vienna, Austria). For all samples, qPCR of the target gene expression was compared to the housekeeping gene (*PPIA*[19]). PCR analysis was performed in triplicate using 50 ng sample per reaction and 200 nM primers (*CXCL8* sense: 5′-ACT GAG AGT GAT TGA GAG TGG AC-3′, *CXCL8* anti-sense: 5′-AAC CCT CTG CAC CCA GTT TTC-3′, *PPIA* sense: 5′-CCA CCA GAT CAT TCC TTC TGT AGC-3′, *PPIA* anti-sense: 5′-CTG CAA TCC AGC TAG GCA TGG-3′)[19] was performed under the following conditions: denaturation 95 °C/20 s, annealing 45 cycles at 95 °C/3 s and 60 °C/30 s and extension 95 °C/15 s, followed by a melting curve: 60 °C for 1 minute, stepwise increase by 0.5 °C to 95 °C for a final 15 seconds. Using LinRegPCR, the PCR efficiency was determined and used to calculate the amount of template at the beginning (N_0-value). The N_0-values of *CXCL8* were normalized to the N_0-values of PPIA. These normalized values were compared to the respective solvent control (set to 1.0) at each time point, resulting in the fold change of treatment *vs.* control.

Determination of LPS-stimulated IL-8 release from HGF-1 cells pre- or concomitantly treated with pitanga juice and pitange juice constituents

For the determination of IL-8 release, 12 000 HGF-1 cells per well were seeded in a 24-well plate five days before the experiment. Cells were exposed to the desired compounds, which were diluted in medium, to reach the concentration found in pitanga juice (oxidoselina-1,3,7(11)-trien-8-one: 30 µg ml^{-1}; cyanidin-3-glucoside: 119 µg ml^{-1}),[7] and 10 µg ml^{-1} *PG*-LPS,[17] at 37 °C and 5% CO_2 as described above. Thereafter, the medium was collected in 1.5 ml tubes. Cell debris was removed by centrifugation at 4 °C, 10 000*g* for 10 min. Supernatant was stored at −80 °C until measurement. IL-8 release was analyzed as described above. This experiment was performed with three different passages (18, 20 and 21) using four replicates per passage.

Statistical analysis

The data are presented as mean values ± standard deviation. The unpaired Student's *t*-test was performed to compare the effect of the substance with the LPS-stimulation for the results of the gene expression and the results of the IL-8 secretion from human epithelial cells and HGF-1 cells. One-way ANOVA with Holm–Sidak post-hoc analysis was used to determine time-dependent effects and differences between the compounds.

Results

Anti-inflammatory effects of pitanga juice in human oral gum epithelial cells treated for 10 min

To investigate the effect of pitanga juice, a pilot trial using human oral gum epithelial cells was performed. Six healthy volunteers swirled 100 ml of the juice for 10 min. Gum epithelial cells were harvested with a sterile brush and were then incubated without (Fig. 1A) or with (Fig. 1B) 10 µg ml^{-1} *PG*-LPS for six hours to stimulate the release of IL-8. An increased release of IL-8 by the gum epithelial cells due to LPS-stimulation of 32.6 ± 9.80% was detected in the incubation media (data not shown). The effect of pitanga juice was compared to its respective control treatment, which was then set to 100% (T/CON or T/LPS [%]). Treatment with pitanga juice inhibited the release of IL-8 in non-stimulated cells by 55 ± 8.2% (Fig. 1A). In cells stimulated with *PG*-LPS, pitanga juice reduced IL-8 release by 52 ± 11% (Fig. 1B).

Previously, two most abundant non-volatile and volatile compounds have been quantified in pitanga pulp. Their concentration were determined with 119 µg ml^{-1} cyanidin-3-glucoside and 30 µg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one in

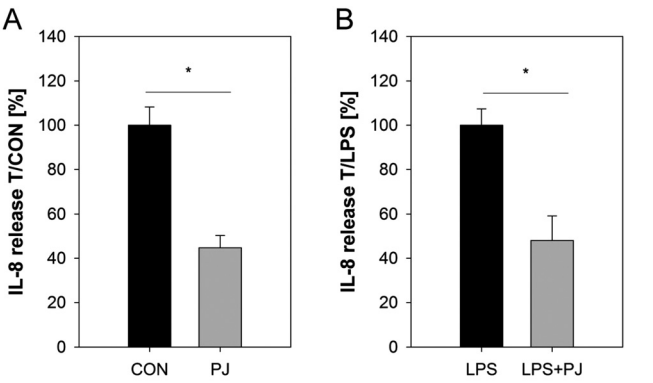

Fig. 1 IL-8 release by human gingival epithelial cells after 6 hours of exposure to pitanga juice (PJ) without (A) or with (B) concomitant stimulation with 10 µg ml^{-1} *PG*-LPS. Non-magnetic bead kit analysis was performed in duplicate using individual cell samples from six volunteers. The Student's *t*-test was applied for calculating significant differences of PJ *vs.* respective control (100%), *$p < 0.05$.

pitanga juice, respectively. Due to limited amounts of oxidoselina-1,3,7(11)-trien-8-one, which had to be isolated from pitanga pulp, performance of an *in vivo* approach using individual compounds to identify their contribution to the anti-inflammatory potential of the juice was not possible. Thus, a well-established cell culture model was chosen to identify effects of the pitanga juice constituents.

Stimulation of *CXCL8* mRNA expression in HGF-1 cells by 10 μg ml^{-1} *PG*-LPS over 24 h

Human gingival fibroblasts (HGF-1), an established model system,[17,20] were chosen to examine the anti-inflammatory potential of pitanga juice constituents. First, a time-course experiment using RT-qPCR was conducted to determine the optimal stimulation period with 10 μg ml^{-1} *PG*-LPS (Fig. 2) on the HGF-1 cells. *CXCL8* expression was normalized against *PPIA* expression and related to non-stimulated control cells (= 1). No influence of *PG*-LPS on the mRNA expression of *CXCL8* was determined after 0.5 and 1 h compared to untreated control cells. After incubation for 3, 6 and 24 hours, a stimulation of *CXCL8* mRNA expression with 10 μg ml^{-1} *PG*-LPS was detected when compared to the untreated control, with a peak present at 3 and 6 h, corresponding to a fold change of 43.8 ± 1.89 and 61.8 ± 8.14 (Fig. 2), respectively.

Cell viability after treatment of HGF-1 cells with pitanga juice and two major constituents thereof

Prior to investigating the anti-inflammatory effects of pitanga juice and the two pitanga constituents, false positive results due to cytotoxic effects should be excluded. Therefore, cell viability was determined by flow cytometry using fluorescent dyes. The viability measured for cells treated with 1% DMSO as a solvent control was set to 100%. A treatment with 10 μg ml^{-1} *PG*-LPS and solvent had no influence on the cell viability compared to the control (data not shown). Furthermore, a pretreatment of HGF-1 cells with pitanga juice or two major constituents thereof, in pitanga juice representative concentrations, did not affect cell viability compared to *PG*-LPS-stimulation (Table 1). In addition, co-incubation of the HGF-1 cells with 10 μg ml^{-1} *PG*-LPS in combination with either 119 μg ml^{-1} cyanidin-3-glucoside or 30 μg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one for 6 h did not decrease HGF-1 cell viability compared to a treatment with LPS and solvent. In contrast, the cell viability was decreased by 49 ± 11% after a concomitant treatment of HGF-1 cells with pitanga juice and *PG*-LPS (Table 1).

Table 1 HGF-1 cell viability after treatment with pitanga juice and juice constituents for 10 min followed by 6 h stimulation with *PG*-LPS (pre-incubation) or concomitantly with compound and LPS for 6 h (co-incubation). Data is related to a solvent control (1% DMSO, control = 100%) and presented as mean treatment over control (T/C) ± SD (n = 3)

	Cell viability (T/C in %)	
Treatment with	Pre-incubation	Co-incubation
10 μg ml^{-1} *PG*-LPS + 1% DMSO	96 ± 8.0[a]	101 ± 5.9[a]
10 μg ml^{-1} *PG*-LPS + 119 μg ml^{-1} C3G	93 ± 15[a]	92 ± 6.8[a]
10 μg ml^{-1} *PG*-LPS + 30 μg ml^{-1} OxS	96 ± 7.1[a]	97 ± 10[a]
10 μg ml^{-1} *PG*-LPS + pitanga juice	91 ± 5.5[a]	51 ± 11[b]

One-way ANOVA with [a,b]$p < 0.01$, *PG*-LPS: lipopolysaccharides from *P. gingivalis*, C3G: cyanidin-3-glucoside, OxS: oxidoselina-1,3,7(11)-trien-8-one.

Immune-modulatory effects of pretreatment with pitanga juice and two major constituents thereof on LPS-stimulated IL-8 release in HGF-1 cells

To simulate the conditions used in the *ex vivo* experiment using human gum epithelial cells, HGF-1 cells were treated for 10 min with pitanga juice, 119 μg ml^{-1} cyanidin-3-glucoside or 30 μg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one, followed by a 6 h stimulation with 10 μg ml^{-1} *PG*-LPS (Fig. 3). *PG*-LPS-stimulation for 6 h resulted in a 39.8 ± 5.02 fold increased release of IL-8 (data not shown). Dexamethasone served as an anti-inflammatory control compound, reducing the *PG*-LPS stimulated *CXCL8* gene expression to 33 ± 8.4% (Fig. 3A) and IL-8 release to 34 ± 8.9% (Fig. 3B), compared to pretreatment with the solvent only. Both, 30 μg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one and 119 μg ml^{-1} cyanidin-3-glucoside, concentrations found in pitanga juice, remained ineffective in inhibiting the *PG*-LPS stimulated *CXCL8* mRNA expression and IL-8 release from HGF-1 cells, when incubated for 10 min prior to the stimulation. In contrast, HGF-1 cells that were pretreated with pitanga juice before stimulation with 10 μg ml^{-1} *PG*-LPS for 6 h, expressed 49 ± 13% less *CXCL8* mRNA (Fig. 3A) and released 76.4 ± 8.34% less IL-8 protein (Fig. 3B) than the stimulated control.

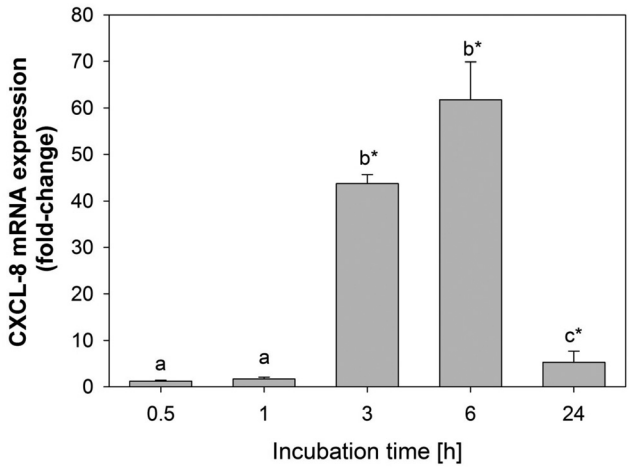

Fig. 2 Time-dependent *CXCL8* mRNA expression by HGF-1 cells after a 0.5, 1, 3, 6 and 24 hour stimulation with 10 μg ml^{-1} *PG*-LPS (n = 3). Student's t-test for significant differences of *PG*-LPS treatment *vs.* control (control = 1) at each time point, *$p < 0.05$; one-way ANOVA with Holm–Sidak post-hoc analysis for influence of time dependent effects (a–c).

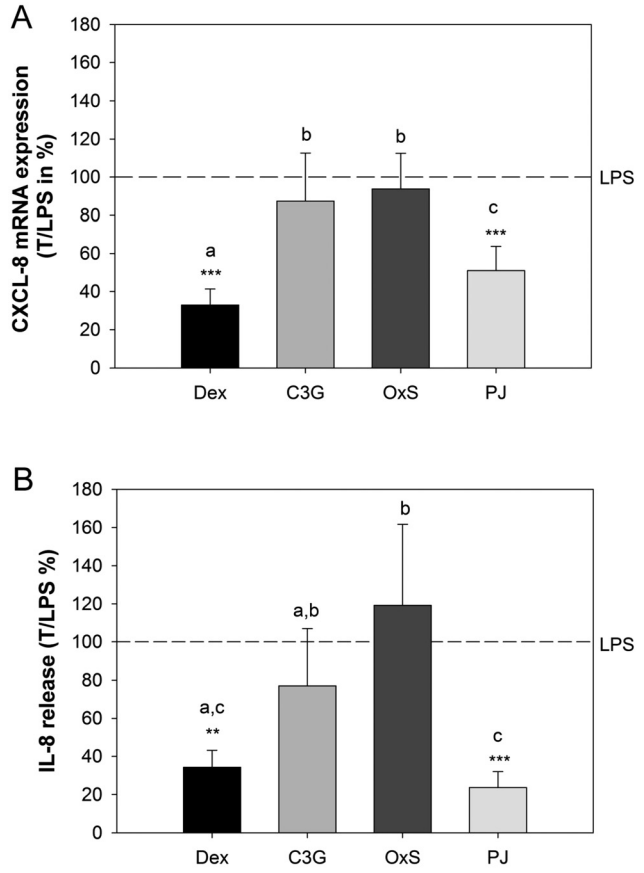

Fig. 3 *CXCL8* mRNA expression (A) and IL-8 release (B) by HGF-1 cells after 10 min exposure to dexamethasone (Dex, 100 nM), cyanidin 3-glucoside (C3G, 119 μg ml^{-1}), oxidoselina-1,3,7(11)-trien-8-one (OxS, 30 μg ml^{-1}) or pitanga juice (PJ), followed by 6 h stimulation with 10 μg ml^{-1} *PG*-LPS (n = 4). Effect of *PG*-LPS alone = 100% (dashed line). Student's t-test against LPS treatment with $^{**}p < 0.01$, $^{***}p < 0.001$, $^{a-c}$one-way ANOVA with $p < 0.05$ between treatments.

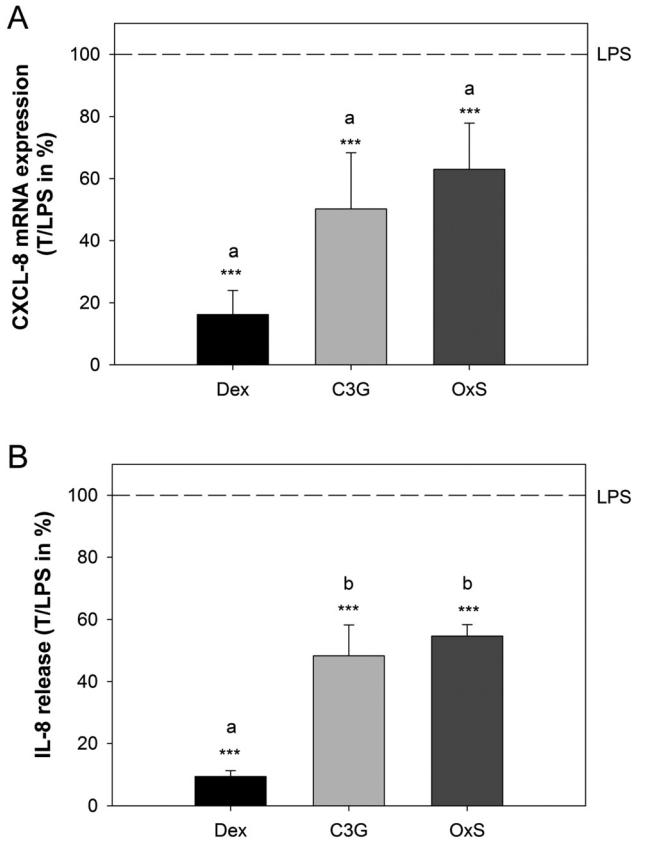

Fig. 4 *CXCL8* mRNA expression (A) and IL-8 release (B) by HGF-1 cells after 6 hour concomitant incubation with 10 μg ml^{-1} *PG*-LPS and dexamethasone (Dex, 100 nM), cyanidin 3-glucoside (C3G, 119 μg ml^{-1}) or oxidoselina-1,3,7(11)-trien-8-one (OxS, 30 μg ml^{-1}), n = 3–4, effect of *PG*-LPS alone = 100% (dashed line). Student's t-test against *PG*-LPS treatment with $^{***}p < 0.001$, (A) ANOVA on ranks due to lack of normality, (B)^{a,b} one-way ANOVA with Holm–Sidak post-hoc analysis, $p < 0.05$ between treatments.

Anti-inflammatory effects of pitanga constituents during concomitant stimulation with *PG*-LPS for 6 h in HGF-1 cells

To investigate whether the pitanga volatile oxidoselina-1,3,7(11)-trien-8-one and non-volatile cyanidin-3-glucoside exploit an anti-inflammatory potential, *PG*-LPS-stimulated HGF-1 cells were concomitantly incubated with either compound for six hours. Treatment of each compound at pitanga juice representative concentrations with 10 μg ml^{-1} *PG*-LPS resulted in an anti-inflammatory activity. *CXCL8* mRNA expression decreased by 50 ± 15% after 6 h upon incubation with cyanidin-3-glucoside and 37 ± 18% when incubated with oxidoselina-1,3,7(11)-trien-8-one (Fig. 4A). Dexamethasone served as a known anti-inflammatory control and reduced the *PG*-LPS stimulated *CXCL8* mRNA expression by 84 ± 8.0% at a concentration of 100 nM after 6 h of concomitant treatment.

Furthermore, the secretion of IL-8 by HGF-1 cells was investigated after concomitant treatment of 10 μg ml^{-1} *PG*-LPS and either 119 μg ml^{-1} cyanidin-3-glucoside or 30 μg ml^{-1} oxidoselina-1,3,7(11)-trien-8-one. The anti-inflammatory control, dexamethasone, attenuated the *PG*-LPS-induced IL-8 release to 9.48 ± 1.85%. Both cyanidin-3-glucoside and oxidoselina-1,3,7(11)-trien-8-one were less effective than dexamethasone but inhibited the *PG*-LPS stimulated release of IL-8 by 52 ± 9.9% and 45 ± 3.7%, respectively (Fig. 4B).

Discussion

Treatment of inflammatory diseases and prevention of gingivitis and periodontitis using plant extracts or rinses are common in traditional medicines.[21,22] While pitanga leaf extracts have been shown to elicit an anti-inflammatory potential, only limited data are available on the anti-inflammatory properties of the consumed product, pitanga juice. We hypothesized that pitanga juice possesses an anti-inflammatory potential with regards to inflammatory diseases of the oral cavity. In addition, the presented work was aimed at evaluating the impact of the main volatile compound and non-volatile compound, which were previously identified by our group.[7]

Commonly, there are two possible research strategies regarding anti-inflammatory potential, whether there is (a) a

preventive effect or (b) a therapeutic effect. These questions can be addressed using different experimental settings. To identify a preventive effect, the cells are pre-treated with the compound of interest followed by stimulation of an immune response. In contrast, a therapeutic effect can be determined using concomitant treatment of inflammatory stimulant and potential anti-inflammatory compound.[19] Evaluation of anti-inflammatory properties may be analyzed through the reduction of cytokine and chemokine secretion in response to an inflammatory stimulus, *e.g.* LPS.[13,19,23] An anti-periodontal activity of extracts or compounds is characterized by the reduction of cytokine release in established cell models of periodontal diseases, *e.g.* human gingival fibroblasts,[17,20] concomitantly treated with both, a stimulant and the compound of interest.

A recent *in vivo* study on 84 subjects with different periodontal health states underlined the crucial role of IL-8 and TNF-α in the progression of periodontal diseases.[24] In addition, previous results from our group identified IL-8 as the most potent marker responding to stimulation with 10 μg ml^{-1} LPS from *Porphyromonas gingivalis*.[17] Bodet *et al.*[25] showed that an anti-inflammatory impact of a cranberry extract on cytokine and chemokine release was greatest regarding IL-8, supporting the choice of IL-8 as a biomarker. To gain an initial insight into the anti-inflammatory potential of pitanga juice, the mRNA expression and release of IL-8 was determined in this study.

In a previous study, stimulation of human gingival fibroblasts with 1 μg ml^{-1} LPS from *E. coli* resulted in an up-regulation of *CXCL8* of 5.26 fold compared to the non-stimulated control after 24 h,[26] supporting the presented data of a 5.3 ± 2.4 fold increase in *CXCL8* mRNA expression after 24 h. However, stimulation of HGF-1 cells with 10 μg ml^{-1} *PG*-LPS resulted in a peak *CXCL8* mRNA expression after 6 h. In addition, IL-8 release was shown to be significantly increased after this time, supporting previous results.[17]

To test a preventive effect against an inflammatory stimulus, pretreatment with the compound of interest for up to 2 h followed by LPS-stimulation up to 24 h has been used in macrophages to identify an anti-inflammatory effect of individual compounds or extracts.[19] Fruit-based extracts may exploit anti-inflammatory properties *in vivo*[27] and *in vitro*,[25,28] and may possess preventive effects.[8] A cranberry extract produced from cranberry juice completely inhibited LPS-stimulated IL-8 release from gingival fibroblasts in a concentration of 50 μg ml^{-1}.[25] In both test systems used in the presented study, oral gum epithelial cells (Fig. 1) and human gingival fibroblasts (Fig. 3), a 10 min treatment with pitanga juice resulted in a decrease of *PG*-LPS-stimulated IL-8 release, indicating a preventive anti-inflammatory potential of pitanga juice. In contrast to using a concentrated extract based on juice,[25] here, pitanga juice contained 35% pitanga pulp, which may explain different efficacies. However, an anti-inflammatory effect was detected using juice prepared after a recipe according to Brazilian law, representing common serving conditions.

It has been shown that topical treatment of gingivitis with a green tea gel for 4 weeks reduced periodontal markers including probe depth by 2.06 ± 0.07 mm or gingival index to a healthy range (<2).[29] The authors based these effects on the anti-inflammatory potential of catechins.[29] The here presented study focused on revealing the contribution of the main volatile compound, oxidoselina-1,3,7(11)-trien-8-one and non-volatile compound cyanidin-3-glucoside,[7] to the anti-inflammatory potential of pitanga juice. Cyanidin-3-glucoside administered to mice exploited an anti-inflammatory effect *in vivo*, reducing the carrageenan-induced cyclooxygenase-2 activity and prostaglandin E_2 release.[30] The anthocyanin cyanidin-3-glucoside is found in different berries, including pitanga and black currant. Desjardins *et al.*[8] found an attenuation of *E. coli*-LPS-stimulated release of IL-6 by macrophages after a 2 hour pre-treatment with black current extract followed by 24 h of stimulation. A black-currant extract and cyanidin-3-glucoside in concentrations of 5 and 25 μg ml^{-1} possessed similar effects. Here, human gingival cells were chosen as a model system for periodontal cells and cells were treated with cyanidin-3-glucoside for only 10 min to simulate the *in vivo* situation of drinking juice, followed by 6 h stimulation with *PG*-LPS. The individual pitanga compounds did not elicit anti-inflammatory effects under these conditions. One limitation of the presented study was that only the two quantitatively dominant compounds were tested. In a previous study, investigating the anti-inflammatory potential of sage tea and using sage tea representative concentrations of the test compounds, we showed that of 3.5 μg ml^{-1} 1,8-cineole significantly decreased the release of IL-8 by HGF-1 cells, while rosmarinic acid (49.1 μg ml^{-1}) did not possess an anti-inflammatory activity.[18] Thus, minor compounds may contribute to the anti-inflammatory activity of pitanga juice.

To test whether the two major pitanga constituents may possess therapeutic anti-inflammatory potential *in vitro*, different test conditions were chosen. The individual compounds did not possess cytotoxic properties in HGF-1 cells in the tested concentrations when applied for 10 min before *PG*-LPS-stimulation or 6 h concomitantly with 10 μg ml^{-1} *PG*-LPS, excluding false positive results. Concomitant treatment of HGF-1 cells with 119 μg ml^{-1} cyanidin-3-glucoside and 10 μg ml^{-1} *PG*-LPS for 6 h, led to a decreased *CXCL8* mRNA expression and IL-8 release (Fig. 4), indicating an anti-inflammatory potential of the anthocyanin at pitanga juice representative concentrations on human gingival fibroblasts. In contrast to the individual pitanga constituents, treatment of HGF-1 cells with pitanga juice for 6 h resulted in a significant reduction of cell viability (Table 1). Thus, no results were obtained for the *CXCL8* mRNA expression and IL-8 release after 6 h concomitant treatment of HGF-1 cells with pitanga juice and 10 μg ml^{-1} *PG*-LPS. One reason of the detected cytotoxic effect of pitanga juice after 6 h of incubation might be the acidic pH of the incubation solution of pH 4.04 ± 0.01, which has been shown to induce cell death in lung epithelial cells already after 5 min in serum-free medium.[31] However, HGF-1 cells recovered from the 10 min treatment with pitanga

juice solution followed by 6 h in cell culture medium with 10% FBS and 10 μg ml^{-1} *PG*-LPS.

In addition to the effects of the non-volatile anthocyanin, it was shown in the presented study for the first time that the main volatile compound previously quantified in pitanga pulp,[7] oxidoselina-1,3,7(11)-trien-8-one, possesses an anti-inflammatory potential. The test compound reduced IL-8 release and *CXCL8* mRNA expression in HGF-1 cells after concomitantly incubated with *PG*-LPS in a similar manner as cyanidin-3-glucoside (Fig. 4). Both major pitanga juice constituents did not exhibit a preventive anti-inflammatory effect but reduced the LPS-stimulated *CXCL8* mRNA expression and IL-8 release when concomitantly incubated with LPS for 6 h. The lack of a preventive effect suggests that the individual compounds interact with *PG*-LPS directly or with the LPS-binding protein, or inhibit the *PG*-LPS binding to the TLR-2 resulting in the onset of the inflammatory signaling.[32]

In summary, pitanga juice elicit an anti-inflammatory effect by reducing the *PG*-LPS-stimulated release of IL-8, a common biomarker of gingival inflammation, after a 10 min exposure of oral gum epithelial cells with pitanga juice followed by a 6 h stimulation with *PG*-LPS. The same effect was detected in HGF-1 cells for pitanga juice, while the main volatile compound and non-volatile compound remained ineffective under the same conditions. However, the tested individual pitanga constituents, cyanidin-3-glucoside and oxidoselina-1,3,7(11)-trien-8-one, possessed anti-inflammatory properties in juice representative concentrations, when HGF-1 cells were concomitantly treated with one of the compounds and *PG*-LPS. Thus, a contribution of the individual compounds to the anti-inflammatory effect warrants further research.

Acknowledgements

The authors thank the CAPES Foundation, Ministry of Education of Brazil, for providing a doctoral sandwich fellowship for Denise Josino Soares.

References

1 G. L. Filho, V. De Rosso, M. A. A. Meireles, P. T. V. Rosa, A. L. Oliveira, A. Z. Mercadante and F. A. Cabral, Supercritical CO(2) extraction of carotenoids from pitanga fruits (*Eugenia uniflora* L.), *J. Supercrit. Fluids*, 2008, **46**(1), 33–39.

2 A. E. Consolini, O. A. Baldini and A. G. Amat, Pharmacological basis for the empirical use of *Eugenia uniflora* L. (Myrtaceae) as antihypertensive, *J. Ethnopharmacol.*, 1999, **66**(1), 33–39.

3 O. Figueiroa Ede, L. C. Nascimento da Silva, C. M. de Melo, J. K. Neves, N. H. da Silva, V. R. Pereira and M. T. Correia, Evaluation of antioxidant, immunomodulatory, and cytotoxic action of fractions from *Eugenia uniflora* L. and *Eugenia malaccensis* L.: correlation with polyphenol and flavanoid content, *Sci. World J.*, 2013, **2013**, 125027.

4 E. E. S. Schapoval, S. M. Silveira, M. L. Miranda, C. B. Alice and A. T. Henriques, Evaluation of Some Pharmacological Activities of Eugenia-Uniflora L, *J. Ethnopharmacol.*, 1994, **44**(3), 137–142.

5 G. Schmeda-Hirschmann, C. Theoduloz, L. Franco, E. Ferro and A. R. de Arias, Preliminary pharmacological studies on Eugenia uniflora leaves: xanthine oxidase inhibitory activity, *J. Ethnopharmacol.*, 1987, **21**(2), 183–186.

6 M. Bagetti, E. M. P. Facco, J. Piccolo, G. E. Hirsch, D. Rodriguez-Amaya, C. N. Kobori, M. Vizzotto and T. Emanuelli, Physicochemical characterization and antioxidant capacity of pitanga fruits (*Eugenia uniflora* L.), *Cienc. Tecnol. Aliment.*, 2011, **31**(1), 147–154.

7 D. Josino Soares, M. Pignitter, M. M. Ehrnhoefer-Ressler, J. Walker, I. Montenegro Brasil and V. Somoza, Identification and quantification of oxidoselina-1,3,7(11)-trien-8-one and cyanidin-3-glucoside as the main volatile and non-volatile constituents in pitanga pulp, *J. Sci. Food Agric.*, 2014, submitted.

8 J. Desjardins, S. Tanabe, C. Bergeron, S. Gafner and D. Grenier, Anthocyanin-rich black currant extract and cyanidin-3-O-glucoside have cytoprotective and anti-inflammatory properties, *J. Med. Food*, 2012, **15**(12), 1045–1050.

9 A. Mauray, C. Felgines, C. Morand, A. Mazur, A. Scalbert and D. Milenkovic, Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice, *Genes Nutr.*, 2010, **5**(4), 343–353.

10 S. G. Lee, B. Kim, Y. Yang, T. X. Pham, Y. K. Park, J. Manatou, S. I. Koo, O. K. Chun, *et al.*, Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-kappaB independent of NRF2-mediated mechanism, *J. Nutr. Biochem.*, 2014, **25**(4), 404–411.

11 T. Ara, K. Kurata, K. Hirai, T. Uchihashi, T. Uematsu, Y. Imamura, K. Furusawa, S. Kurihara, *et al.*, Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease, *J. Periodontal Res.*, 2009, **44**(1), 21–27.

12 D. L. Wolf and I. B. Lamster, Contemporary concepts in the diagnosis of periodontal disease, *Dent. Clin. North Am.*, 2011, **55**(1), 47–61.

13 O. Andrukhov, S. Ertlschweiger, A. Moritz, H. P. Bantleon and X. Rausch-Fan, Different effects of P. gingivalis LPS and *E. coli* LPS on the expression of interleukin-6 in human gingival fibroblasts, *Acta Odontol. Scand.*, 2013, **72**(5), 337–345.

14 R. P. Darveau, T. T. Pham, K. Lemley, R. A. Reife, B. W. Bainbridge, S. R. Coats, W. N. Howald, S. S. Way, *et al.*, Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4, *Infect Immun.*, 2004, **72**(9), 5041–5051.

15 G. M. Gu, M. D. Martin, R. P. Darveau, E. Truelove and J. Epstein, Oral and serum IL-6 levels in oral lichen planus patients, *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.*, 2004, **98**(6), 673–678.

16 T. D. Herath, R. P. Darveau, C. J. Seneviratne, C. Y. Wang, Y. Wang and L. Jin, Tetra- and penta-acylated lipid A struc-

tures of Porphyromonas gingivalis LPS differentially activate TLR4-mediated NF-kappaB signal transduction cascade and immuno-inflammatory response in human gingival fibroblasts, *PLoS One*, 2013, **8**(3), e58496.

17 J. M. Walker, A. Maitra, J. Walker, M. M. Ehrnhoefer-Ressler, T. Inui and V. Somoza, Identification of Magnolia officinalis L. bark extract as the most potent anti-inflammatory of four plant extracts, *Am. J. Chin. Med.*, 2013, **41**(3), 531–544.

18 M. M. Ehrnhoefer-Ressler, K. Fricke, M. Pignitter, J. M. Walker, J. Walker, M. Rychlik and V. Somoza, Identification of 1,8-cineole, borneol, camphor, and thujone as anti-inflammatory compounds in a Salvia officinalis L. infusion using human gingival fibroblasts, *J. Agric. Food Chem.*, 2013, **61**(14), 3451–3459.

19 J. Walker, K. Schueller, L. M. Schaefer, M. Pignitter, L. Esefelder and V. Somoza, Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations, *Food Funct.*, 2014, **5**(1), 74–84.

20 Z. Dzierzewicz, J. Szczerba, J. Lodowska, D. Wolny, A. Gruchlik, A. Orchel and L. Weglarz, The role of Desulfovibrio desulfuricans lipopolysaccharides in modulation of periodontal inflammation through stimulation of human gingival fibroblasts, *Arch. Oral Biol.*, 2010, **55**(7), 515–522.

21 T. Ara, Y. Maeda, Y. Fujinami, Y. Imamura, T. Hattori and P. L. Wang, Preventive effects of a Kampo medicine, Shosaikoto, on inflammatory responses in LPS-treated human gingival fibroblasts, *Biol. Pharm. Bull.*, 2008, **31**(6), 1141–1144.

22 V. Nanayakkara and L. Ekanayake, Use of traditional medicine for oral conditions in rural Sri Lanka, *Int. Dent. J.*, 2008, **58**(2), 86–90.

23 H. W. Cheng, K. C. Lee, K. P. Cheah, M. L. Chang, C. W. Lin, J. S. Li, W. Y. Yu, E. T. Liu, *et al.*, Polygonum viviparum L. inhibits the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages through haem oxygenase-1 induction and activation of the Nrf2 pathway, *J. Sci. Food Agric.*, 2013, **93**(3), 491–497.

24 A. S. Ertugrul, H. Sahin, A. Dikilitas, N. Alpaslan and A. Bozoglan, Comparison of CCL28, interleukin-8, interleukin-1beta and tumor necrosis factor-alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis, *J. Periodontal Res.*, 2013, **48**(1), 44–51.

25 C. Bodet, F. Chandad and D. Grenier, Cranberry components inhibit interleukin-6, interleukin-8, and prostaglandin E production by lipopolysaccharide-activated gingival fibroblasts, *Eur. J. Oral Sci.*, 2007, **115**(1), 64–70.

26 Y. G. Choi, S. Yeo, S. H. Kim and S. Lim, Anti-inflammatory changes of gene expression by Artemisia iwayomogi in the LPS-stimulated human gingival fibroblast: microarray analysis, *Arch. Pharm. Res.*, 2012, **35**(3), 549–563.

27 D. Gupta, D. J. Bhaskar, R. K. Gupta, B. Karim, V. Gupta, H. Punia, M. Batra, A. Jain, *et al.*, Effect of Terminalia chebula Extract and Chlorhexidine on Salivary pH and Periodontal Health: 2 Weeks Randomized Control Trial, *Phytother. Res.*, 2013, **28**(7), 992–998.

28 C. Bodet, F. Chandad and D. Grenier, Inhibition of host extracellular matrix destructive enzyme production and activity by a high-molecular-weight cranberry fraction, *J. Periodontal Res.*, 2007, **42**(2), 159–168.

29 V. K. Chava and B. D. Vedula, Thermo-reversible green tea catechin gel for local application in chronic periodontitis: a 4-week clinical trial, *J. Periodontol.*, 2013, **84**(9), 1290–1296.

30 N. M. Hassimotto, V. Moreira, N. G. do Nascimento, P. C. Souto, C. Teixeira and F. M. Lajolo, Inhibition of carrageenan-induced acute inflammation in mice by oral administration of anthocyanin mixture from wild mulberry and cyanidin-3-glucoside, *Biomed. Res. Int.*, 2013, **2013**, 146716.

31 Q. Chen, Y. Huang, Y. Yang and H. Qiu, Acid induced cell injury and death in lung epithelial cells is associated with the activation of mitogen activated protein kinases, *Mol. Med. Rep.*, 2013, **8**(2), 565–570.

32 Y. Zhang and X. Li, Lipopolysaccharide-regulated production of bone sialoprotein and interleukin-8 in human periodontal ligament fibroblasts: the role of toll-like receptors 2 and 4 and the MAPK pathway, *J. Periodontal Res.*, 2014, DOI: 10.1111/jre.12193.

