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Ellagitannins modulate the inflammatory response of human neutrophils *ex vivo*

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ABSTRACT

Background: Tannin-rich plant materials are commonly used in the traditional medicine as external anti-inflammatory, antioxidant and antimicrobial agents. Plant extracts containing significant quantities of tannins are often used in the prevention and treatment of oral cavity diseases such as periodontosis or gingivitis. The contribution of pure ellagitannins to the observed anti-inflammatory activity of tannin-rich remedies is still not resolved.

Purpose: The aim of the present study the study was to establish if ellagitannins and their precursor – pentagalloylglucose (**1**) can modulate the inflammatory response of *ex-vivo* stimulated neutrophils.

Methods: Human neutrophils were isolated from the buffy coats obtained from healthy volunteers. Neutrophils were cultivated with or without tested compounds. The influence of ellagitannins and **1** on the production and release of pro-inflammatory factors such as elastase, reactive oxygen species, interleukin-8, tumour necrosis factor alpha (TNF-α) and metalloproteinase-9 was evaluated using ELISA sets or chemical methods. The effect on surface expression of toll like receptor 4 (TLR-4) and apoptosis was also checked using flow cytometry.

Results: The results showed that ellgitannins modulate the inflammatory response of human neutrophils by the inhibition of production and release of chosen cytokines and pro-inflammatory enzymes. By the induction of TNF-α ellagitannins enhance neutrophil apoptosis, which is of interest in the case of chronic inflammation within oral cavity. Ellagitannins also decrease the surface expression of TLR-4 in activated neutrophils.

Conclusion: The results support the traditional use of tannin-rich products in the prevention and treatment of oral cavity diseases. The present study proves the substantial contribution of ellagitannins to the anti-inflammatory activity of tannin-rich medicinal plant materials.

Introduction

Ellagitannin-rich plant materials are commonly used in the traditional medicine as anti-inflammatory, antioxidant and antimicrobial agents (Evans 2009; Lipińska et al. 2014; Piwowarski et al. 2014b). Most of current applications of tannin-rich plant drugs are associated with external use. Infusions containing high concentration of ellagitannins prepared from oak bark (*Quercus spp.*), wood avens rhizome (*Geum urbanum* L.), common agrimony herb (*Agrimonia eupatoria* L.) or tormentil rhizome (*Potentilla erecta* (L.) Raeusch.) are often used topically in skin diseases or as therapeutic rinses and mouthwashes in order to threat bacterial infection and reduce the inflammation (Kumar et al. 2015; Menković et al. 2011; Tomczyk and Latté 2009). The tannin-rich extracts like *Punica granatum* pericarp extract or *Quercus alba* bark extract are also used as active ingredients in herbal toothpastes used in everyday hygiene of oral cavity. Tannic acid which consists of hydrolysable tannins, mainly pentagalloylglucose (penta-*O*-galloyl-β-D-glucose - **1**) and its derivatives, was used as a major ingredient of toothpaste used in the treatment of teeth and to prevent tooth decay (Hirota et al. 1986). Herbal mixtures containing oak bark and/or tormentil rhizome are often used in gums inflammations (Salam et al. 2015; Tomczyk and Latté 2009). The observed effect of ellagitannin-rich plant extract is often connected with non-specific astringent, antimicrobial or hemostatic properties of this group of compounds (Evans 2009). The contribution of pure ellagitannins to the anti-inflammatory activity of plant extracts is still not resolved.

Neutrophils, which belong to the polymorphonuclear cell family (PMNs) are the most abundant group of white cells in human blood. They are a crucial part of the innate immune system. Neutrophils are mobilized from bone marrow and migrate to the place of infection where they are responsible for inflammatory response of the host. Neutrophils can be activated by exogenous factors like components of bacterial wall (lipopolysaccharide (LPS)) or endogenous factor like interleukin 8 (IL-8). Activated neutrophils generate huge

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amounts of reactive oxygen species (ROS) including superoxide ion (O_2^-), hydrogen peroxide and hypochlorous acid (HClO). They also secrete several cytokines (TNF-α, IL-8, IL-1β) and extracellular matrix degrading enzymes such as elastase and matrix metalloproteinases - 2 and – 9 (MMP-2 and 9). Those factors are mainly responsible for neutrophils migration and microbial destruction (Cascao et al. 2009; Mocsai 2013; Tintinger et al. 2013; Witko-Sarsat et al. 2000).

Dental caries, periodontal and gingival tissues diseases are the main dental pathologies affecting humankind. These conditions are caused by plaque formed by Gram-negative bacteria (Rosas-Pinon et al. 2012). It has been shown that the mucosal efflux of neutrophils plays an essential role in the generation and progression of inflammatory response of the host against bacteria (Galgut et al. 2001). On the other hand the chronic over-activation of neutrophils results in host tissue damage and is believed to be responsible for the development of destructive phases of periodontal and gingival diseases (Galgut et al. 2001). The hyperactivated neutrophils are responsible for the host tissue damage and instead of host defense create condition favorable for pathogenic bacteria to grow and invade (Nussbaum and Shapira 2011; Scott and Krauss 2012).

Previous research on the bioactivity of ellgitannins and ellagitannin-rich extracts revealed that the observed anti-inflammatory effect might be explained by the specific inhibition of some pro-inflammatory enzymes or inhibition of molecular signaling path responsible for the initiation and progression of inflammation rather than through non-specific interactions. Some monomeric and dimeric ellagitannins were proven to inhibit hyaluronidase and metalloproteinase-2/-9 activity (Lee et al. 1993; Tanimura et al. 2005). Oenothein B isolated from *Epilobium angustifolium*, which is a dimeric macrocyclic ellagitannins was proven to inhibit hyaluronidase and elastase activity as well as was being able to inhibit the production or secretion of some pro-inflammatory cytokines in *in vitro* studies (Kiss et al. 2011; Schmid et al. 2012). Similar properties were proven for monomeric and dimeric ellagitannins purified from *Lythrum salicaria* (Piwowarski and Kiss 2015). In contrast Hrenn et al. (2006) showed that dimeric ellagitannin agrimoniin (which is a dominating polyphenol in *Agrimoniae herba* and *Tormentillae rhizoma*) and monomeric pedunculagin (present in *Geum urbanum, Agrimoniae herba* and *Tormentillae rhizoma*) caused the direct inhibition of neutrophil elastase but did not influence the release of this proteolitic enzyme from stimulated cells. Other studies indicated that coriariin A, agrimoniin but not their precursor PGG were able modulate the inflammatory response of PBMC through the elevation of TNF-α levels (Feldman et al. 1999). Immunostimulatory effects were also observed by Kolodziej et al. (2001) for monomeric, dimeric and trimeric ellagitannins using RAW 264.7 macrophages cell line.

The aim of the present study the study was to establish if ellagitannins and their precursor – pentagalloylglucose (**1**) can modulate the inflammatory response of *ex-vivo* stimulated neutrophils. Basing on the results obtained for ellagitannins with different molecular structures the contribution of this class of polyphenols to the anti-inflammatory activity of plant extracts used in the prevention and treatment of oral cavity diseases is discussed.

Materials and methods

Chemicals

Camptothecin (98% purity), luminol, f-MLP (formyl-met-leu-phenylalanine), SAAVNA (N-succinyl-alanine-alanine-valinine-p-nitroanilide), cytochalasin B, TMB (3,3',5, 5'-tetramethylbenzidine) liquid substrate system, Hanks' balanced salt solution (HSSB), L-glutamine, fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and RPMI 1640 medium were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). LPS (lipopolysacharide) was purchased from Merck Millipore (Billerica, MA, USA). Curcumin (**C**) and quercetin (**Q**) (>95% purity) were purchased from Carl Roth (Karlsruhe, Germany). Propidium iodide was purchased from BD Biosciences (San Diego, CA, USA). All substances used were of > 95% purity. Phosphate-buffered saline (PBS) was purchased from Gibco (Carlsbad, CA, USA).

Ellagitannins and PGG

Compounds used in the present study were isolated previously from chosen plant materials. Penta-*O*-galloyl-β-D-glucose (**1**) was isolated from defatted seed of *Oenothera paradoxa* Hudziok (Kiss et al. 2008). 1-*O*-galloyl-4,6-(S)-HHDP-β-D-glucose (**2**), pedunculagin (**3**), stachyurin (**4**), casuarinin (**5**), stenophyllanin A (**8**), gemin G (**10**) and gemin A (**11**) were isolated from root of *Geum urbanum* L. (Piwowarski et al. 2014a). Vescalagin (**6**), castalagin (**7**), salicarinin A (**13**) and salicarinin B (**14**) were purified from aerial parts of *Lythrum salicaria* L. (Piwowarski and Kiss 2013). Oenothein B (**9**) was isolated from aerial parts of *Oenothera hoelsheri* Renner ex Rostanski (Granica and Kiss 2012). Agrimoniin (**12**) was obtained from aerial parts of *Agrimonia eupatoria* L. (Granica et al. 2013). All compounds used were of > 95% HPLC-DAD-MS purity. Before each experiment compounds were dissolved in deionized water or in 70% ethanol (v/v) in the case of PGG and then diluted to obtain stock solutions of 400 μM and filtrated through 0.22 μm sterile syringe filter (Carl Roth). Compounds were stored in the darkness at 4 °C and were used for no longer than 72 h. All compounds were stable in experimental conditions.

Isolation of human neutrophils

The buffy coat was prepared from peripheral venous blood collected from healthy human donors (20–35 years old) at the Warsaw Blood Donation Center. All Donors declared that they were non-smokers and did not take any medications. They were confirmed to be healthy and all tests carried out showed values within a normal range. Neutrophils were isolated using a standard method by dextran sedimentation and centrifugation in a Ficoll Hypaque gradient (Böyum 1968). The purity of neutrophils preparation was over 97%. After isolation, cells were suspended in HBSS, PBS or culture medium and maintained at 4 °C before use.

Evaluation of ROS production by human neutrophils

The ROS production by f-MLP-stimulated neutrophils was determined using luminol-dependent chemiluminescence. The concentrations of compounds used in the experiment were 1, 5 and 20 μM. Following isolation, cells were suspended in HBSS. Cell suspension (3.0×10^5) was incubated with 50 μl of the samples with tested compounds in the proper concentration and luminol in a 96-well plate. ROS production was initiated by the addition of f-MLP (0.1 μg/ml) to obtain a total volume of 200 μl/well. Changes in chemiluminescence were measured over a 40 min period at intervals of 2 min in a microplate reader (BioTek). The values obtained in the point corresponding to the maximum of chemiluminescence were taken for calculations. Background chemiluminescence produced by non-stimulated cells was also determined. As a positive control, quercetin was used at concentration of 10 μM. The percentage of ROS production was calculated in comparison to the control without investigated compounds, taking into account chemiluminescence emission inhibited by tested compounds.

Evaluation of elastase release by human neutrophils

Neutrophil elastase release was determined using SAAVNA as a substrate, and p-nitrophenol was measured spectrophotometrically