



# Contribution of C-glucosidic ellagitannins to *Lythrum salicaria* L. influence on pro-inflammatory functions of human neutrophils

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**Abstract** The herb *Lythrum salicaria* L. (Lythraceae) is used in traditional medicine to treat diseases with an inflammatory background, such as haemorrhoidal disease, dysentery, chronic intestinal catarrh, eczema, varicose veins, periodontosis and gingivitis. Because these diseases are closely associated with an excessive inflammatory response of stimulated neutrophils, the influence of aqueous extract and isolated C-glucosidic ellagitannins (dimeric salicarinins A, B and C, vescalagin, castalagin) on their pro-inflammatory functions was examined. *Lythrum salicaria* aqueous extract was shown to modulate lipopolysaccharide (LPS)-triggered production of IL-8 (at 20 µg/mL, 16.6 ± 4.2 % inhibition) but had no influence on MMP-9 production. It was active towards cytochalasin A/f-MLP-stimulated elastase release (at 20 µg/mL, 21.5 ± 3.9 % inhibition), myeloperoxidase release (at 1 µg/mL, 26.5 ± 5.4 % inhibition) and f-MLP- and PMA-induced reactive oxygen species production (at 20 µg/mL, 67.0 ± 3.9 and 66.5 ± 1.9 % inhibition, respectively). The extract was also shown to inhibit expression of integrin CD11b on the neutrophil surface without influencing selectin CD62L shedding. Dose-dependent inhibition of hyaluronidase activity was observed with IC<sub>50</sub> = 10.1 ± 1.2 µg/mL. The main C-glucosidic ellagitannins were shown to be responsible for all these activities with more significant participation attributable to dimeric

salicarinins A, B, C. This study has demonstrated potent activity of aqueous extract on stimulated neutrophils; this enhanced response is known to cause pathological changes in skin and mucosa tissues. These observations support and explain the traditional use of the herb *Lythrum salicaria* to treat certain diseases with an inflammatory background. C-glucosidic ellagitannins, especially dimeric salicarinins, are the factors responsible for these effects.

**Keywords** *Lythrum salicaria* · Ellagitannins · Neutrophils · Inflammation

## Introduction

The herb *Lythrum salicaria* L. has been used in traditional medicine to treat diseases with an inflammatory background, such as haemorrhoidal disease, dysentery, chronic intestinal catarrh, eczema, varicose veins, periodontosis and gingivitis [1–4]. These diseases are closely associated with an excessive inflammatory response and increased extracellular matrix degradation causing impaired tissue integrity. Neutrophils, which enhance the response, are known to cause pathological changes in skin and mucosa tissues and are important contributors to the development of pathological changes during progression of the above diseases. Being responsible for the innate immune system defensive response to microorganisms, neutrophils generate huge amounts of reactive oxygen species (ROS), including superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hypochlorous acid (HClO). Neutrophils also secrete cytokines such as IL-8 and enzymes degrading extracellular matrix (ECM) elastase and matrix metalloproteinases. These factors are mainly responsible for neutrophil infiltration and microbial destruction. On the other

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hand, the prolonged over-activation of neutrophils (due to exogenous bacterial factors such as LPS or due to endogenous IL-8 produced by local fibroblasts) results in host tissue damage and is believed to contribute to the development of destructive phases of diseases with an inflammatory background [5–8].

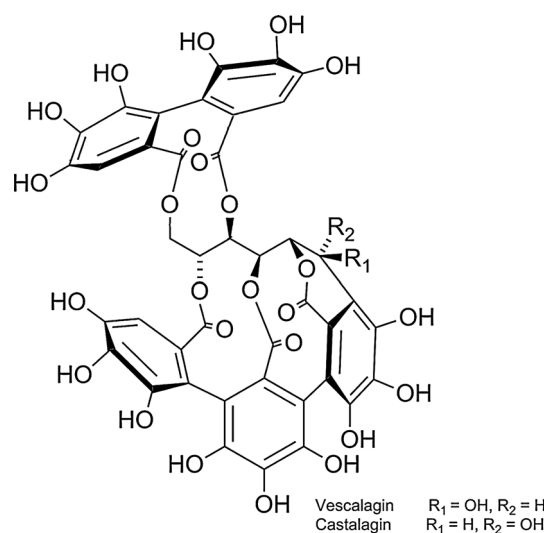
*Lythrum salicaria* belongs to the Lythraceae family. The main compounds on which pharmacopoeial standardization is based (according to the European Pharmacopoeia, 8th edition) are tannins. *C*-glucosidic monomeric (vescalagin and castalagin) and dimeric (salicarinins A, B, C) ellagitannins have been shown to be the dominant compounds in aqueous extracts [9, 10]. Other phenolics, such as *C*-glucosidic flavonoids, orientin, vitexin, isovitexin and iso-orientin, were also detected. In previous studies, the presence of phenolic acids, mainly ellagic acid together with phthalates and sterols, has been described [11]. Pawlaczyk et al. [12] isolated acidic glycoconjugate with pro-coagulant activity. However, since the *C*-glucosidic ellagitannins are significantly dominant, they were targeted in the present study as the compounds likely to be producing the aqueous extract's bioactivity.

Because of excessive neutrophil infiltration and release of pro-inflammatory cytokines, enzymes degrading ECM and ROS are important in the development of inflammatory diseases of the skin and mucosa, and the *ex-vivo* model using neutrophils isolated from human peripheral venous blood was chosen to explain the beneficial effects of extracts of *Lythrum salicaria* and to evaluate the participation of ellagitannins in the observed activity.

## Materials and methods

### Plant material and chemicals

The *Lythrum salicaria* flowering herb was collected from its natural habitat in Umer in the Świętokrzyskie region (Poland) in July 2011. Its identity was confirmed anatomically and morphologically based on its pharmacopoeial monograph in the Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, where the voucher specimen UMER2011 is deposited. Preparation of an aqueous extract (LSH) and isolation of ellagitannins: vescalagin (V), castalagin (C), salicarinin A (SA), salicarinin B (SB) and salicarinin C (SC) (Figs. 1, 2) from *Lythrum salicaria* was performed according to Piwowarski and Kiss [9]. The identity of the ellagitannins was confirmed by NMR and MS spectra. Isolated ellagitannins were of >95 % purity (determined by the HPLC–DAD–CAD method). Camptothecin (98 % purity), luminol, PMA (4 $\beta$ -phorbol-12 $\beta$ -myristate-R13-acetate), f-MLP (formyl-met-leu-phenylalanine), ascorbic acid (reagent grade), curcumin, SAAVNA (*N*-succinyl-alanine-alanine-valinine-*p*-



**Fig. 1** Structures of monomeric ellagitannins: vescalagin and castalagin

nitroanilide), LPS, cytochalasin A, TMB (3,3',5, 5'-tetramethylbenzidine) liquid substrate system, Triton X-100, Hanks' balanced salt solution (HBSS) and RPMI 1640 medium, bovine testis hyaluronidase and hyaluronic acid sodium salt from *Streptococcus equi* sp. were purchased from Sigma–Aldrich GmbH (Steinheim, Germany). Lucigenin and quercetin (>95 % purity) were purchased from Carl Roth (Karlsruhe, Germany). Gallic acid (>96 % purity) was purchased from ChromaDex (Santa Ana, USA). 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).

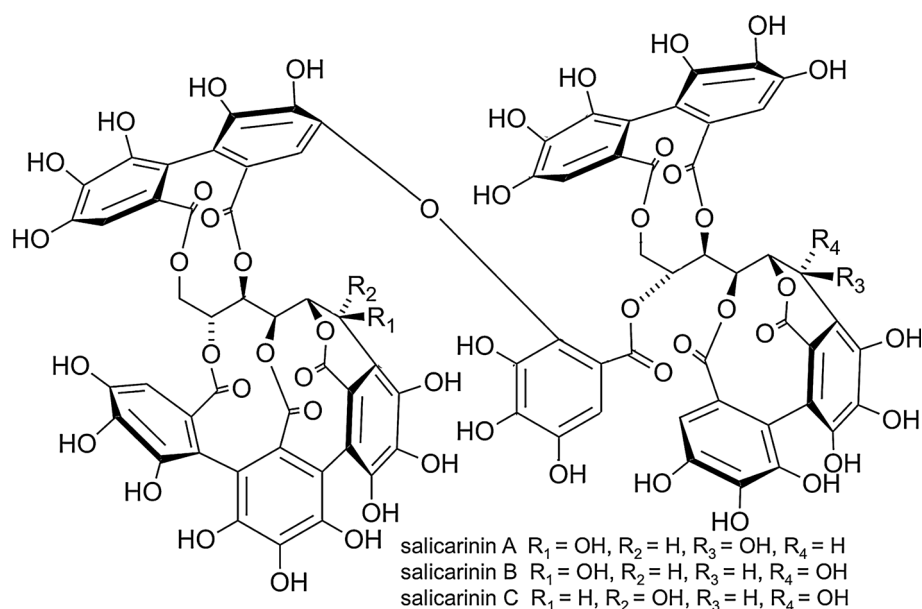
### Isolation of human neutrophils

Peripheral venous blood was taken from healthy human donors (20–35 years old) in the Warsaw Blood Donation Centre. Donors did not smoke and were not taking any medication. They were clinically confirmed to be healthy, and routine laboratory tests showed values within the normal ranges. The study conformed to the principles of the Declaration of Helsinki. Neutrophils were isolated using a standard method of dextran sedimentation prior to hypotonic lysis of erythrocytes and to centrifugation in a Ficoll Hypaque gradient. The purity of neutrophil preparations was >97 %, and viability measured by trypan blue exclusion was >98 %. Neutrophils were then resuspended in appropriate medium to perform the selected assay.

### Cytotoxicity

Cytotoxicity of the extract and ellagitannins was determined by standard flow cytometric probe using propidium

**Fig. 2** Structures of dimeric ellagitannins: salicarinin A, salicarinin B and salicarinin C



iodide (PI) staining to distinguish cells with diminished membrane integrity according to the method previously described by Shinella et al. [13]. For short-term cytotoxicity determination, neutrophils ( $4 \times 10^5/\text{mL}$ ) were incubated in PBS with extract at a concentration of  $20 \mu\text{g}/\text{mL}$  and compounds at a concentration of  $20 \mu\text{M}$  for 1.5 h. For long-term cytotoxicity, neutrophils ( $2 \times 10^5/\text{mL}$ ) were cultured in a 24-well plate in RPMI 1640 medium with 10 % FBS, 10 mM HEPES, and 2 mM L-glutamine for 24 h at  $37^\circ\text{C}$  with 5 %  $\text{CO}_2$  in the presence of extract at a concentration of  $20 \mu\text{g}/\text{mL}$  and compounds at concentrations of  $20 \mu\text{M}$ . After 24 h, the neutrophils were harvested and centrifuged ( $1500 \text{ RPM}$ ; 10 min;  $4^\circ\text{C}$ ), washed once with cold PBS and resuspended in  $400 \mu\text{L}$  PBS.  $5 \mu\text{L}$  of PI ( $50 \mu\text{g}/\text{mL}$ ) solution was added to the cell suspension. After 15 min of incubation at room temperature, cells were analysed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA); 10,000 events were recorded per sample. Cells that displayed high permeability to PI were expressed as a percentage of PI(+) cells. Camptothecin at a concentration of  $10 \mu\text{M}$  was used as a positive control.

#### IL-8 and MMP-9 production

Neutrophils ( $2 \times 10^5/\text{mL}$ ) were cultured in 24-well plates in RPMI 1640 medium with 10 % FBS, 10 mM HEPES, and 2 mM L-glutamine in the absence or presence of LPS ( $100 \text{ ng}/\text{mL}$ ) for 24 h at  $37^\circ\text{C}$  with 5 %  $\text{CO}_2$  in the absence or presence of extract at concentrations of 1, 5 and  $20 \mu\text{g}/\text{mL}$ , and compounds at concentrations of 1, 5 and  $20 \mu\text{M}$  were added to 1 mL of cell suspension 1 h before the stimuli. After 24 h, the neutrophils were harvested and

centrifuged ( $2000 \text{ RPM}$ ; 10 min;  $4^\circ\text{C}$ ). The amount of IL-8 or MMP-9 released into cell supernatants was measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). PBS was used as a non-stimulated control. Curcumin was used as a positive control, according to Aggarwal and Harikumar [14] and Antoine et al. [15].

#### Expression of adhesion molecules CD62L and CD11b/CD18

The influence of urolithins at the level of adhesion molecules on the neutrophil surface was determined using the flow cytometric method.  $500 \mu\text{L}$  of cell suspension ( $1 \times 10^6$ ) in PBS buffer was incubated with extract at concentrations of 1, 5 and  $20 \mu\text{g}/\text{mL}$  or compound at concentrations of 1, 5 and  $20 \mu\text{M}$  for 30 min at  $37^\circ\text{C}$  prior to 30 min stimulation with  $10 \mu\text{L}$  of cytochalasin A ( $5 \mu\text{g}/\text{mL}$ ) and f-MLP ( $0.1 \mu\text{g}/\text{mL}$ ). Neutrophils were marked with monoclonal antibody against CD62L-(APC)-conjugate (Becton–Dickinson) or CD11b-(PE)-conjugate (Becton–Dickinson) and incubated for 30 min at  $4^\circ\text{C}$  in the dark. The cells were analysed by flow cytometry FACSCalibur (Becton–Dickinson) and data from 20,000 events were recorded.

#### Elastase release

Neutrophil elastase release was determined using SAAVNA as a substrate, and *p*-nitrophenol was measured spectrophotometrically.  $100 \mu\text{L}$  of cell suspension ( $5 \times 10^5/\text{mL}$ ) in HBSS was preincubated with  $50 \mu\text{L}$  of compound or extract

solution (final concentrations of 1, 5 and 20  $\mu\text{M}$  or  $\mu\text{g/mL}$ ) for 15 min at 37 °C and then stimulated with 50  $\mu\text{L}$  of cytochalasin A (5  $\mu\text{g/mL}$ ) and f-MLP (0.1  $\mu\text{g/mL}$ ) for 15 min. The neutrophils were centrifuged (2000 rpm; 10 min; 4 °C). After the addition of 50  $\mu\text{L}$  of SAAVNA solution (1.6 mg/mL) to 100  $\mu\text{L}$  of supernatant, the extent of *p*-nitrophenol was measured spectrophotometrically for 1 h at intervals of 20 min, at 412 nm using a microplate reader (BioTek). According to Kanashiro et al. [16], quercetin was used as a positive control.

#### Myeloperoxidase (MPO) release

Neutrophil MPO release was determined using TMB as a substrate. The assay is based on the oxidation of TMB by MPO in the presence of  $\text{H}_2\text{O}_2$  [17]. 100  $\mu\text{L}$  of cell suspension ( $2 \times 10^5/\text{mL}$ ) in HBSS was preincubated with 50  $\mu\text{L}$  of compound or extract solution (final concentrations of 1, 5 and 20  $\mu\text{M}$  or  $\mu\text{g/mL}$ ) for 15 min at 37 °C and then stimulated with 50  $\mu\text{L}$  of cytochalasin A (5  $\mu\text{g/mL}$ ) and f-MLP (0.1  $\mu\text{g/mL}$ ) for 15 min. After centrifugation (2000 rpm; 10 min; 4 °C), 100  $\mu\text{L}$  of supernatant was incubated with 50  $\mu\text{L}$  of the TMB liquid substrate system. The reaction was terminated after 20 min by the addition of 2 M hydrochloric acid. The absorbance was measured at 655 nm using a microplate reader (BioTek). Gallic acid was used as a positive control, according to Kroes et al. [18].

#### ROS production

Generation of oxidants by f-MLP or PMA-stimulated neutrophils was measured using luminol- or lucigenin-dependent chemiluminescence tests. 70  $\mu\text{L}$  of cell suspension ( $2 \times 10^5/\text{mL}$ ) in HBSS was incubated with 50  $\mu\text{L}$  of compound or extract solution (final concentrations of 1, 5 and 20  $\mu\text{M}$  or  $\mu\text{g/mL}$ ) together with 50  $\mu\text{L}$  of luminol (100  $\mu\text{M}$ ) or lucigenin (200  $\mu\text{M}$ ) solution. ROS production was initiated by the addition of 30  $\mu\text{L}$  of f-MLP (0.1  $\mu\text{g/mL}$ ) or PMA (1  $\mu\text{g/mL}$ ). Changes in chemiluminescence at 37 °C were measured immediately for 45 min at intervals of 2 min in a microplate reader (Biotek). Ascorbic acid was used as a positive control.

#### Hyaluronidase activity

The influence of extract and ellagitannins on hyaluronidase activity was measured using the USP turbidimetric method modified by Piwowarski et al. [19]. Heparin was used as a positive control.

#### Statistical analysis

The results are presented as mean values  $\pm$  SEM of the indicated number of experiments. Statistical significance of

differences between means was determined by one-way ANOVA. For comparison of results with the control group, Dunnett's post hoc test was used. To compare the differences between the inhibitory activities of compounds, Tukey's post hoc test was performed. Results with a *p* value  $< 0.05$  were considered statistically significant. All analyses were performed using Statistica 10 software.

## Results

### Cytotoxicity

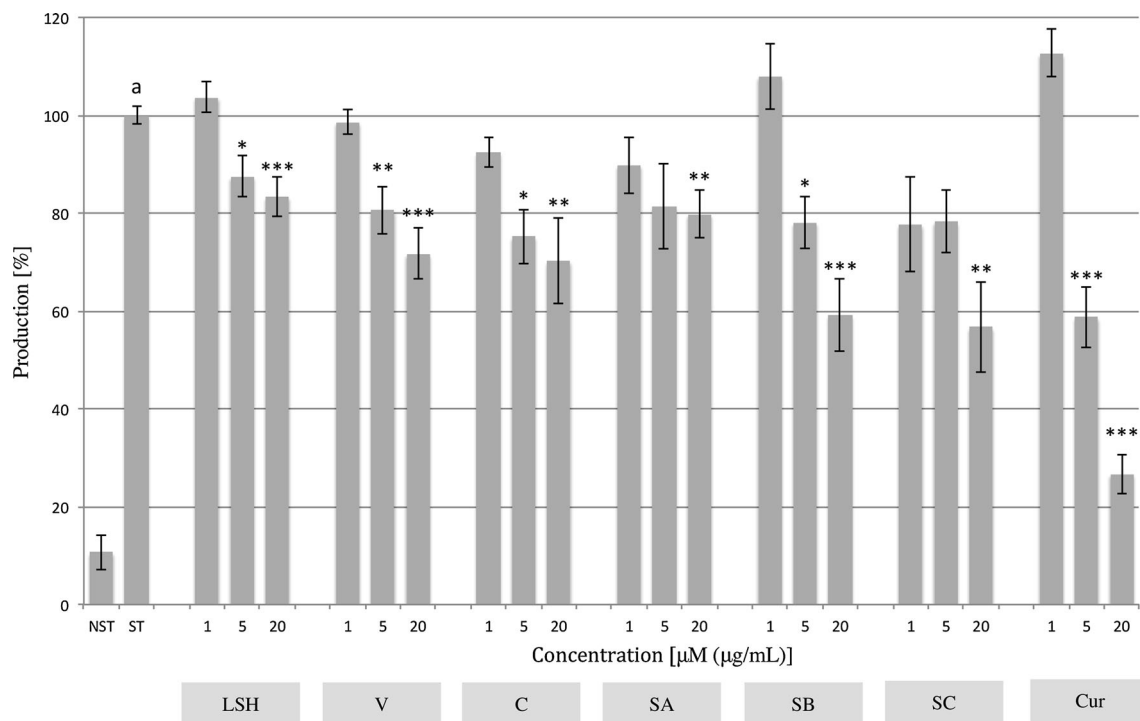
*Lythrum salicaria* aqueous extract (LSH) as well as isolated ellagitannins at tested concentrations did not show any cytotoxic effect on neutrophils in either long- or short-term tests (Figs. S1, S2).

### IL-8 and MMP-9 production

The influence of LSH and ellagitannins on the inflammatory response of neutrophils triggered by stimulation of Toll-like receptor-4 (TLR-4) by LPS was examined. LSH exhibited a moderate inhibitory effect towards IL-8 production (Fig. 3; Table S1). However, statistically significant inhibition of production was observed at concentrations of 5 and 20  $\mu\text{g/mL}$  ( $12.5 \pm 4.3$  and  $16.6 \pm 4.2$  %, respectively). Ellagitannins seem to be responsible for the observed inhibition, as all tested compounds have shown activity at a concentration of 20  $\mu\text{M}$ . LSH at tested concentrations had no effect on production responsible for ECM degradation MMP-9 (Fig. S3; Table S2). However dimeric ellagitannins SB and SC were slightly active at a concentration of 20  $\mu\text{M}$  ( $21.2 \pm 4.1$  and  $13.4 \pm 3.7$  %, respectively). The effects of extract and ellagitannins on IL-8 and MMP-9 production were compared with curcumin used as a positive control.

### Expression of adhesion molecules, CD62L and CD11b/CD18

The rolling and the firm adhesion of the neutrophils to the endothelium leading to their transendothelial migration to the inflammation site are crucial events of inflammatory state progression. When the pro-inflammatory factor occurs, shedding of the molecule responsible for rolling, selectin (CD62L), is observed, while the level of the molecule providing firm adhesion, integrin (CD11b), becomes enhanced on the neutrophil surface [20]. LSH and ellagitannins have shown dose-dependent inhibition of CD11b expression triggered by cytochalasin A/f-MLP (Fig. 4). The effects of LSH were observed even at a concentration of 1  $\mu\text{g/mL}$ . The greater contribution of



**Fig. 3** Effect of *Lythrum salicaria* aqueous extract (LSH) at concentrations of 1, 5 and 20 µg/mL and vescalagin (V), castalagin (C), salicarinin A, B and C (SA, SB, SC, respectively) at concentrations of 1, 5 and 20 µM on IL-8 production by LPS-stimulated neutrophils. Curcumin (Cur) at concentrations of 1, 5 and 20 µM was used as a positive control. Data were expressed as mean ± SEM of three

separate experiments performed with neutrophils isolated from independent donors assayed in duplicate. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  versus stimulated control (Dunnett's post hoc test); <sup>a</sup>statistically significant ( $p < 0.001$ ) versus non-stimulated control; ST stimulated control, NST non-stimulated control. Values of means, SEM and statistics are provided in Table S1

dimeric ellagitannins to this activity was observed, as SA, SB and SC acted significantly more strongly than monomeric compounds. Neither LSH nor ellagitannins have prevented CD62L from shedding (data not shown).

#### Elastase release

Elastase is a neutrophil serine proteinase responsible for ECM degradation, stored in azurophilic granules ready for immediate release upon stimulation. It increases tissue permeability and eases neutrophil migration to the inflammation site [21]. LSH was shown to inhibit elastase release from cytochalasin A/f-MLP stimulated neutrophils (Fig. 5; Table S3). Statistically significant inhibition was observed at 20 µg/mL ( $21.5 \pm 3.9 \%$ ). Only dimeric ellagitannins, SA, SB and SC, were responsible for the observed effects, all potent to inhibit enzyme release at a concentration of 20 µM ( $57.8 \pm 7.1$ ,  $61.8 \pm 4.5$  and  $49.4 \pm 5.9 \%$  inhibition, respectively). The effect was compared with quercetin used as a positive control. Monomeric ellagitannins V and C were completely inactive at the tested concentrations.

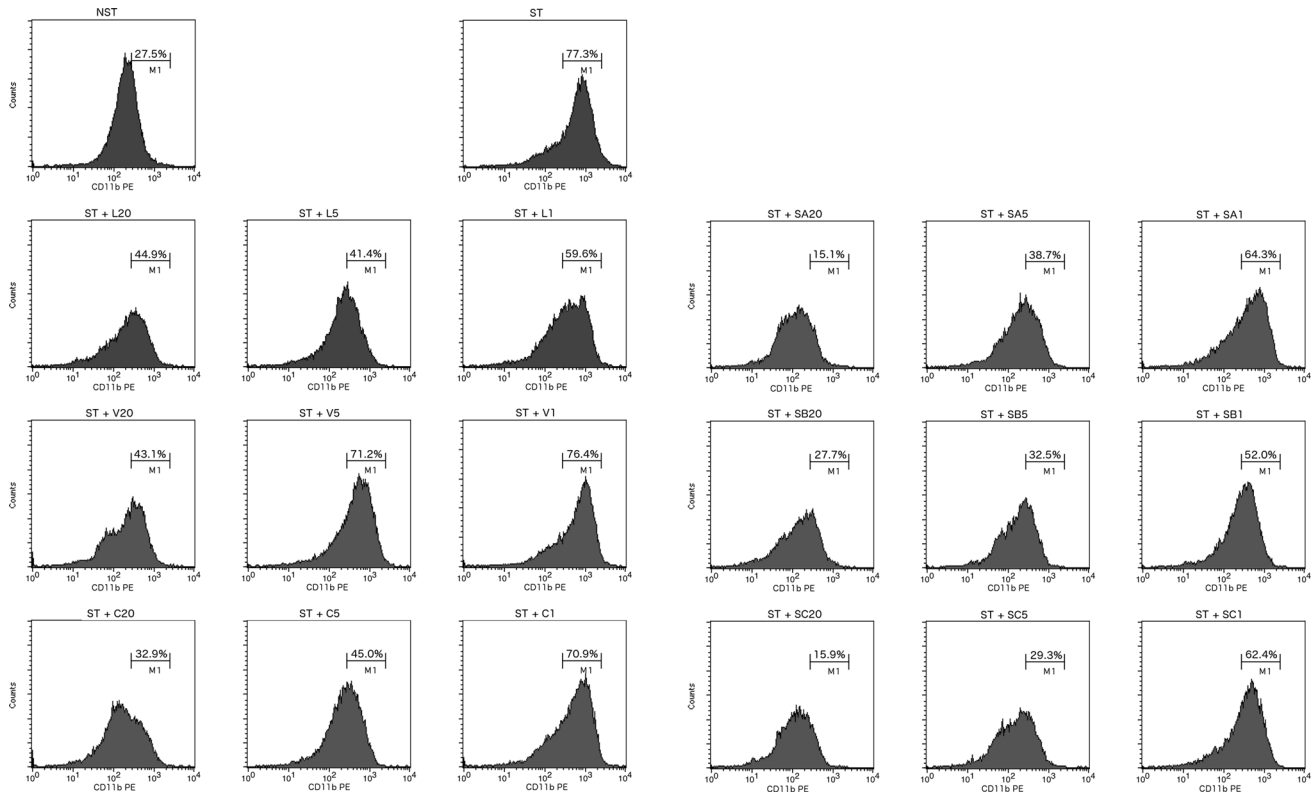
#### MPO release

MPO is another pro-inflammatory ready-to-release enzyme stored in neutrophils' azurophilic granules, being responsible for the production of HClO from H<sub>2</sub>O<sub>2</sub> and Cl<sup>-</sup> during respiratory bursts. LSH inhibited its release even at a concentration of 1 µg/mL ( $26.5 \pm 5.4 \%$  inhibition). Monomeric ellagitannins V and C expressed inhibitory activity at all tested concentrations. However, dimeric compounds SA, SB, and SC produced the highest contribution to this effect, showing inhibition at a concentration of 20 µM of  $98.5 \pm 0.3$ ,  $97.1 \pm 1.5$  and  $94.0 \pm 3.4 \%$ , respectively. Their activity was compared with the known neutrophil MPO release inhibitor, gallic acid (Fig. 6; Table S4).

#### ROS production

The inhibition of ROS release from stimulated neutrophils was determined using two different models. One was with stimulation achieved by bacterial-derived peptide, f-MLP, which leads to generation of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and HClO, and the other was by direct kinase C activator, PMA, which results





**Fig. 4** Effect of *Lythrum salicaria* aqueous extract (LSH) at concentrations of 1, 5 and 20 µg/mL and vescalagin (V), castalagin (C), salicarinin A, B and C (SA, SB, SC, respectively) at concentrations of 1, 5 and 20 µM on integrin CD11b expression on the surface of

cytochalasin A/f-MLP-stimulated neutrophils. ST stimulated control, NST non-stimulated control, M1 histogram marker for stimulated control

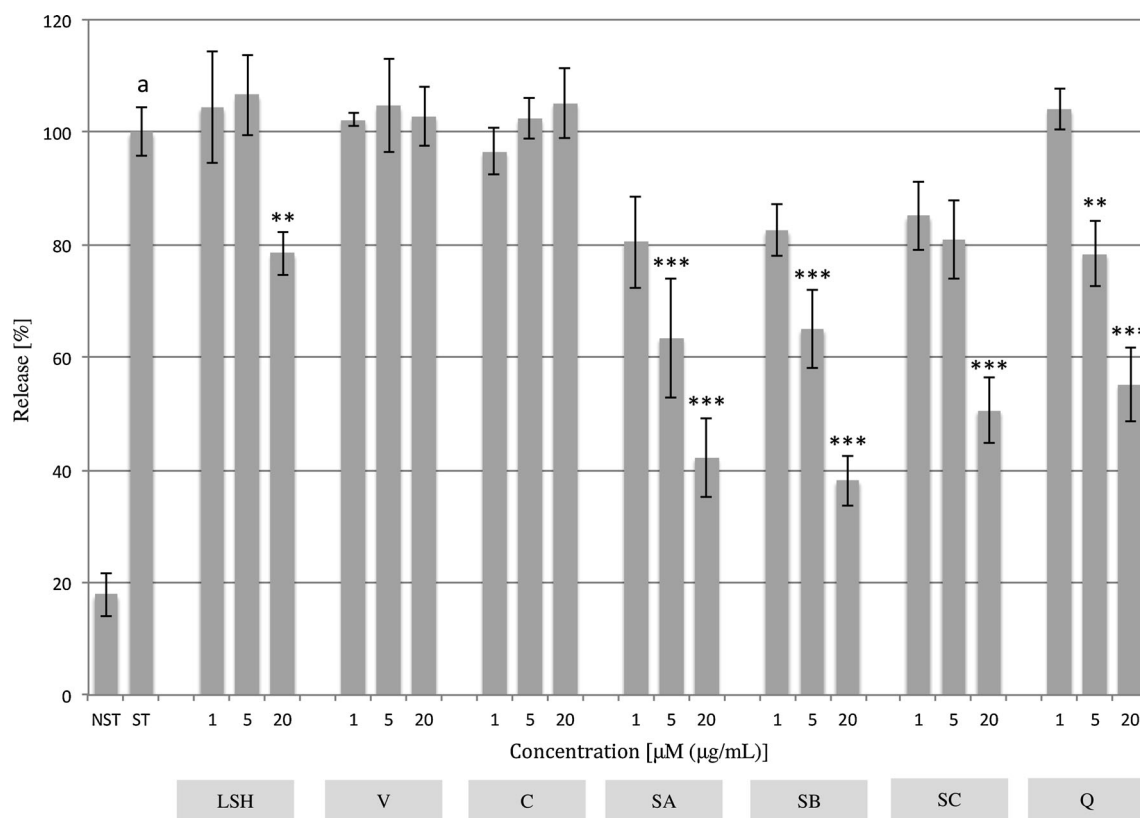
in  $O_2^-$  and  $H_2O_2$  release. LSH inhibited ROS release in a dose-dependent manner at all tested concentrations in both models, acting significantly more strongly than ascorbic acid (Fig. 7; Table S5). Monomeric and dimeric ellagitannins were responsible for the observed inhibition, and the latter were shown to be more potent in these assays.

#### Hyaluronidase activity

Increased hyaluronidase activity is not only responsible for ECM degradation and increased tissue permeability, but also leads to the appearance of a low-molecular-weight hyaluronan which is known to be a strong inflammation inducer [22]. LSH was shown to inhibit hyaluronidase activity in a dose-dependent manner with  $IC_{50} = 10.1 \pm 1.2$  µg/mL (Fig. S4; Table S6), reaching  $94.4 \pm 0.6$  % inhibition at 20 µg/mL. Ellagitannins were established to be responsible for this effect and, as in assays conducted on neutrophil models, dimeric SA, SB and SC ( $IC_{50} = 1.6 \pm 0.1$ ,  $1.6 \pm 0.2$  and  $2.5 \pm 0.2$  µM, respectively) acted more strongly than monomeric V and C ( $IC_{50} = 3.1 \pm 0.2$  and  $3.1 \pm 0.2$  µM, respectively).

#### Discussion

In our previous screening study conducted on tannin-rich plant materials used in Polish traditional medicine, *Lythrum salicaria* aqueous extract was shown to be the most potent for prevention of ECM degradation [19]. Strong inhibition of hyaluronidase activity and elastase release were determined together with primary phytochemical investigations. It was suspected that ellagitannins, which were present in the extract in significant amounts ( $27.4 \pm 0.8$  %), were responsible for these effects. To evaluate this hypothesis, initially the dominating ellagitannins were isolated and identified together with qualitative characterization of extract using the UHPLC–DAD–MS/MS method [9]. Three novel dimeric C-glucosidic ellagitannins, salicarinin A, B and C, were isolated in addition to two known ellagitannins, vescalagin and castalagin. The amount of each ellagitannin in *Lythrum salicaria* was determined using the UHPLC–CAD method to indicate their levels in aqueous extract. Vescalagin, castalagin, salicarinin A and salicarinin C were  $10.22 \pm 0.51$ ,  $7.12 \pm 0.32$ ,  $11.2 \pm 0.52$  and  $9.68 \pm 0.49$  mg/g of raw material, respectively [10].

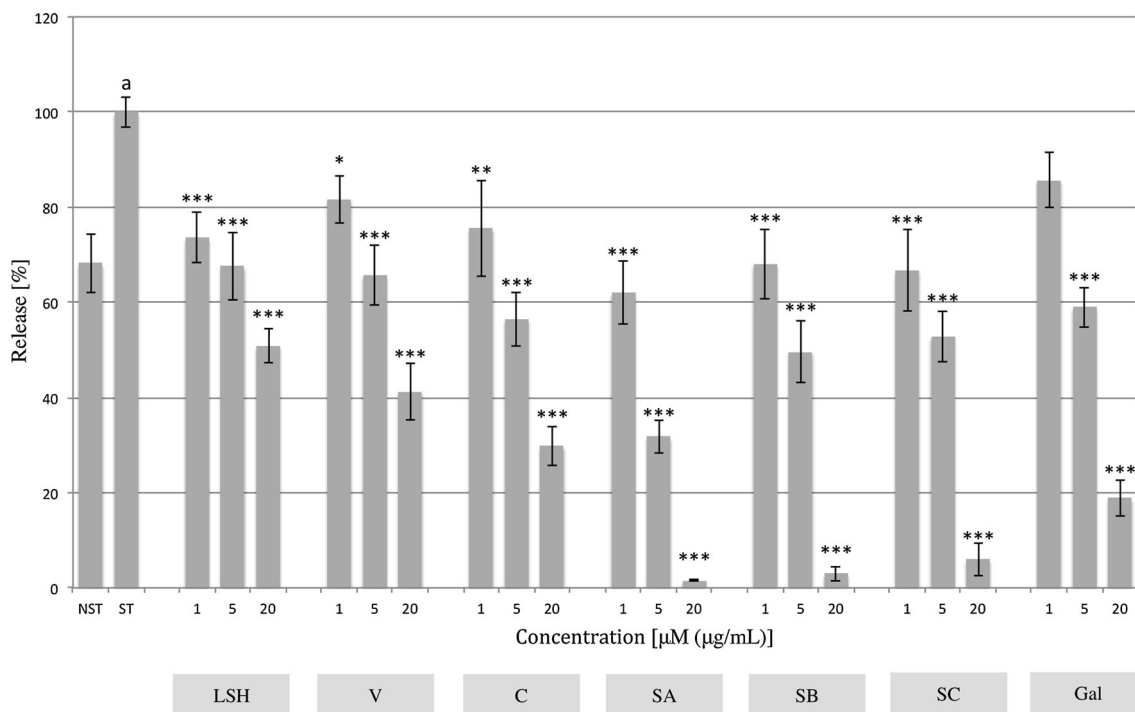


**Fig. 5** Effect of *Lythrum salicaria* aqueous extract (LSH) at concentrations of 1, 5 and 20  $\mu\text{g}/\text{mL}$  and vescalagin (V), castalagin (C), salicarinin A, B and C (SA, SB, SC, respectively) at concentrations of 1, 5 and 20  $\mu\text{M}$  on elastase release from cytochalasin A/f-MLP-stimulated neutrophils. Quercetin (Q) at concentrations of 1, 5 and 20  $\mu\text{M}$  was used as a positive control. Data were expressed as mean  $\pm$  SEM of four separate experiments performed with

neutrophils isolated from independent donors assayed in duplicate. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus stimulated control (Dunnett's post hoc test); <sup>a</sup>statistically significant ( $p < 0.001$ ) versus non-stimulated control; NST non-stimulated control. Values of means, SEM and statistics provided in Table S3

The ex-vivo studies presented here were conducted using the neutrophil model and they revealed potent anti-inflammatory activity of *Lythrum salicaria* for which dominating C-glucosidic ellagitannins were shown to be mainly responsible. According to the results obtained, the dimeric salicarinins have a greater effect than the monomeric vescalagin and castalagin. The observed differences are consistent with previous studies concerned with the structure–activity relationships of ellagitannins, which demonstrated that dimeric compounds have significantly stronger biological effects than monomeric compounds [23]. In the case of elastase release, only dimeric salicarinins were responsible for the extract's inhibitory effect. Hrenn et al. [24] conducted studies on pedunculagin (monomeric ellagitannin) and agrimoniin (dimeric ellagitannin) pointing to stronger elastase inhibition for the latter but in-silico studies led the authors to conclude that this effect occurs in an unspecific manner. Taking into consideration the lack of activity determined for monomeric vescalagin and castalagin compared with the strong activity

of salicarinins, it can be concluded that the C-glucosidic ellagitannins examined here act specifically on neutrophil elastase. In the case of the anti-oxidant and anti-myeloperoxidase activity of *Lythrum salicaria* extract, the larger contribution is attributable also to dimeric salicarinins as, in these assays, their influence was determined to be stronger than that of monomeric compounds. However, inhibition by vescalagin and castalagin was also significantly marked. Similar observations were made for inhibition of CD11b expression as integrin is responsible for the firm adhesion of neutrophils to endothelium, which is the necessary first event for neutrophil transendothelial migration and infiltration to the inflammation site. Moreover, *Lythrum salicaria* extract inhibited hyaluronidase activity in a dose-dependent manner as did all isolated ellagitannins. As in neutrophil model assays, dimeric ellagitannins were more potent than monomeric ones. Inhibition of enzyme is known to be related to increased permeability of vascular and other tissues, together with further enhancement of the inflammatory process which



**Fig. 6** Effect of *Lythrum salicaria* aqueous extract (*LSH*) at concentrations of 1, 5 and 20 µg/mL and vescalagin (*V*), castalagin (*C*), salicarinin A, B and C (*SA*, *SB*, *SC*, respectively) at concentrations of 1, 5 and 20 µM on MPO release from cytochalasin A/f-MLP-stimulated neutrophils. Gallic acid (*Gal*) at concentrations of 1, 5 and 20 µM was used as a positive control. Data were expressed as mean ± SEM of three separate experiments performed with

neutrophils isolated from independent donors assayed in triplicate. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  versus stimulated control (Dunnett’s post hoc test); <sup>a</sup>statistically significant ( $p < 0.001$ ) versus non-stimulated control; *ST* stimulated control, *NST* non-stimulated control. Values of means, SEM and statistics provided in Table S4

can be beneficial from a therapeutic point of view. Taking into consideration the above results, it can be concluded that dimeric salicarinins A, B and C are mainly responsible for the anti-inflammatory activity of *Lythrum salicaria*, together with the weaker, but also active, monomeric vescalagin and castalagin. The orientation of the hydroxyl group at anomeric C1 of the glucose chain had no significant influence on activity either in dimeric or in monomeric compounds.

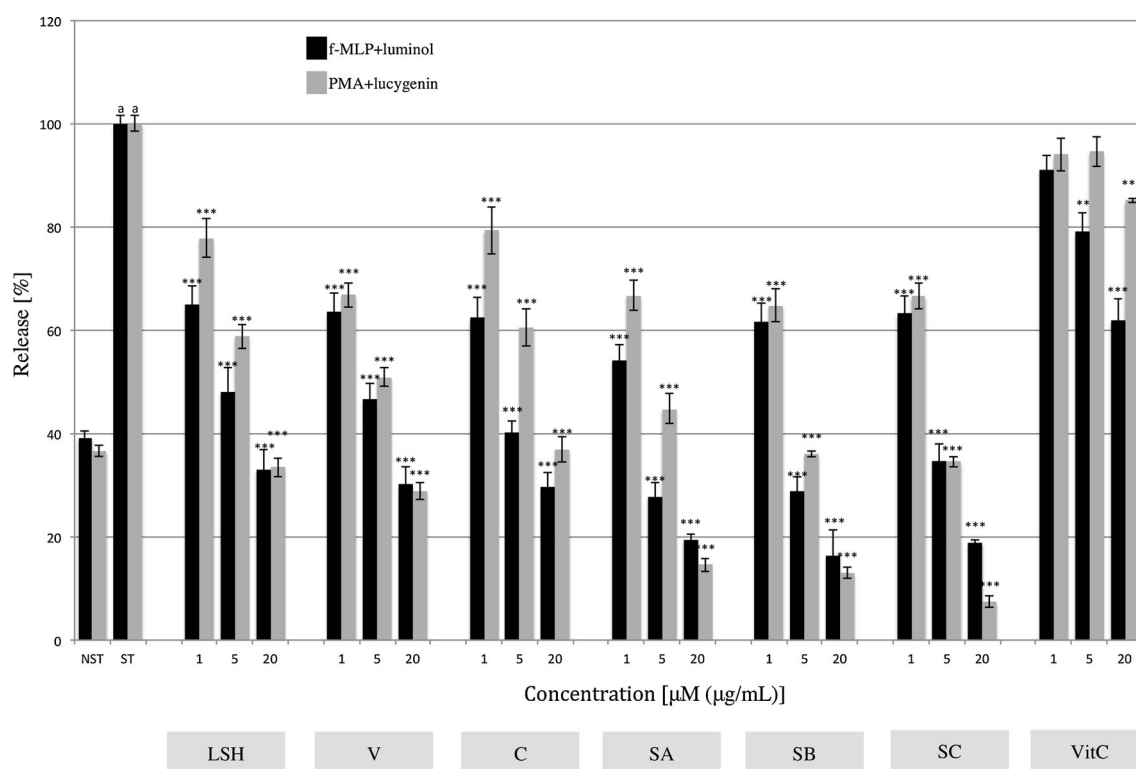
Studies conducted by Tunalier et al. [4] on an in-vivo animal model have shown anti-nociceptive (*p*-benzoquinone-induced abdominal constriction test) and anti-inflammatory (carrageenan-induced hind paw edema model) activity for orally administered polar extracts. The authors pointed out the flavonoids, isovitexin and isoorientin, as compounds responsible for the observed bioactivity. The contribution of ellagitannins needs further investigation due to their problematic bioavailability and their metabolism by gut microbiota to dibenzopyran-6-one derivatives, urolithins [25]. The anti-oxidant in-vitro effects observed on the neutrophil model are consistent with those determined by Tunalier et al. [4].

Castalagin was shown to have a gastroprotective effect in vivo. Administered orally at a dose of 50 mg/kg b.w., it

was very potent in the reduction of ethanol-induced gastric lesions in a mouse model. The authors proposed that the antioxidant properties of tannins could be a mechanism of gastroprotective activity [26]. The influence on neutrophil pro-inflammatory activity determined in the present study could also make an important contribution as their infiltration and activation has been shown to play a part in the development of ethanol-induced gastric lesions [27].

The effects of *Lythrum salicaria* extract and isolated ellagitannins on neutrophil pro-inflammatory activity could explain and support its use in the treatment of diseases for which the development of neutrophil over-activation is responsible. Neutrophils have been shown to participate in the development and progression of various gastrointestinal diseases. Reactive oxygen species derived from neutrophils are implicated in the pathogenesis of oesophageal inflammation induced by the reflux of gastroduodenal contents [28]. Neutrophil elastase is involved in stress-induced gastric mucosal lesion formation by decreasing gastric PGI2 production, which leads to reduced gastric mucosal blood flow and subsequent ischaemic mucosal injury [29]. Elastase and ROS were also indicated as important contributors to the gastric mucosal injury induced by administration of aspirin during *Helicobacter pylori* infection





**Fig. 7** Effect of *Lythrum salicaria* aqueous extract (LSH) at concentrations of 1, 5 and 20 µg/mL and vescalagin (V), castalagin (C), salicarinin A, B and C (SA, SB, SC, respectively) at concentrations of 1, 5 and 20 µM on ROS release from neutrophils upon f-MLP or PMA stimulation detected by luminol or lucygenin, respectively. Ascorbic acid (VitC) at concentrations of 1, 5 and 20 µM was used as a positive control. Data were expressed as mean ± SEM of four

separate experiments performed with neutrophils isolated from independent donors assayed in triplicate. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  versus stimulated control (Dunnett's post hoc test); <sup>a</sup>statistically significant ( $p < 0.001$ ) versus non-stimulated control; ST stimulated control, NST non-stimulated control. Values of means, SEM and statistics provided in Table S5

[30]. It is thus indicated that local pharmacological inhibition of neutrophil elastase could help to prevent gastric mucosal injury of different aetiologies. Neutrophils are also crucial participants in inflammatory bowel disease. It has been shown that strong and specific IL-8 expression in the affected mucosa correlates with the histological grade of active inflammation. Secretion of IL-8 at sites of intestinal inflammation triggers the ongoing recruitment of neutrophils and leads to the release of proteases and ROS responsible for tissue damage and a vicious cycle of neutrophil attraction and activation. Neutrophil trafficking is currently highlighted in designing a therapy for inflammatory bowel disease targeting adhesion molecules, which are crucial for neutrophil intestinal recruitment and retention [31].

The contribution of neutrophils to the development of various skin and mucosa conditions is also underlined. Neutrophil-derived ROS are involved in the disruption of the integrity of the follicular epithelium, which is responsible for the inflammatory processes of acne [32]. Neutrophils play an important role in the pathogenesis of many autoimmune skin diseases by expressing cell-surface receptors and

intracellular signalling molecules. Inhibition of their pro-inflammatory activity is a mechanism of many synthetic anti-inflammatory drugs and is a target for designing new molecules effective for therapy of skin autoimmune diseases [33]. Genetic and experimental data have shown a clear association between neutrophil infiltration into the periodontal tissues and the severity and progression of inflammatory periodontal diseases. Release of chemokines, elastase and ROS leads to vascular endothelium integrity damage, which increases vascular permeability, a cause of further tissue destruction and oedema [34].

The effectiveness of external use of *Lythrum salicaria* in traditional medicine in the treatment of skin and mucosa diseases can be additionally supported by its anti-microbial activity towards *Staphylococcus aureus*, *Proteus mirabilis* and *Micrococcus luteus*, which vescalagin has been shown to be responsible for [35]. Antifungal activity towards *Candida albicans* was also determined [36]. Moreover, the anticoagulant activity attributed to glycoconjugates present in *Lythrum salicaria* [12, 37] can further explain the benefits of its traditional use in such cases as haemorrhoidal disease or periodontitis and gingivitis.

The first complex cell-based anti-inflammatory studies of *Lythrum salicaria* have shown strong activity of the aqueous extract on stimulated neutrophils, the enhanced response of which is known to trigger pathological changes in skin and mucosa tissues. These observations can support and explain the traditional use of *Lythrum salicaria* for external therapy of certain diseases with an inflammatory background. C-glucosidic ellagitannins, especially dimeric salicarinins, were found to be the factors responsible for these effects.

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