# ORCA – Online Research @ Cardiff

# Anti-Inflammatory Activity of *Punica granatum L.* (Pomegranate) Rind Extracts Applied Topically to *ex vivo* Skin

David MJ Houston[a,b], Joachim Bugert[b,c], Stephen P Denyer[a,d], Charles M Heard[a*]

[a] Cardiff School of Pharmacy & Pharmaceutical Science, Cardiff University, Cardiff CF10 3NB Wales, United Kingdom

[b] Department of Microbiology & Infectious Diseases, School of Medicine, Cardiff University, Cardiff CF10 3NB Wales, United Kingdom

[c] Institute of Microbiology, Armed Forces Medical Academy, Neuherbergstraße 11, 80937 München, Germany

[d] University of Brighton, Mithras House, Lewes Road, Brighton BN2 4AT.

Corresponding author. Tel.: +44 2920 8759819. Email address: heard@cardiff.ac.uk (CM Heard)

**Abstract**


Coadministered pomegranate rind extract (PRE) and zinc (II) produces a potent virucidal activity against *Herpes simplex* virus (HSV); however, HSV infections are also associated with localised inflammation and pain. Here, the objective was to determine the anti-inflammatory activity and relative depth penetration of PRE, total pomegranate tannins (TPT) and zinc (II) in skin, *ex vivo*. PRE, TPT and $ZnSO_4$ were dosed onto freshly excised *ex vivo* porcine skin mounted in Franz diffusion cells and analysed for COX-2, as a marker for modulation of the arachidonic acid inflammation pathway, by Western blotting and immunohistochemistry. Tape stripping was carried out to construct relative depth profiles. Topical application of PRE to *ex vivo* skin downregulated expression of COX-2, which was significant after just 6 h, and maintained for up to 24 h. This was achieved with intact stratum corneum, proving that punicalagin penetrated skin, further supported by the depth profiling data. When PRE and $ZnSO_4$ were applied together, statistically equal downregulation of COX-2 was observed when compared to the application of PRE alone; no effect followed the application of $ZnSO_4$ alone. TPT downregulated COX-2 less than PRE, indicating that tannins alone may not be entirely responsible for the anti-inflammatory activity of PRE. Punicalagin was found throughout the skin, in particular the lower regions, indicating appendageal delivery as a significant route to the viable epidermis. Topical application of TPT and PRE had significant anti-inflammatory effects in *ex vivo* skin, confirming that PRE penetrates the skin and modulates COX-2 regulation in the viable epidermis. Pomegranates have potential as a novel approach in ameliorating the inflammation and pain associated with a range of skin conditions, including cold sores and herpetic stromal keratitis.

**Key words** COX-2, *ex vivo*, anti-inflammation, pomegranate, Punica granatum, tannins, skin, tape stripping

**Abbreviations**

| | |
|---|---|
| COX-2 | cyclooxygenase-2 |
| DAB | 3,3'-diaminobenzidine |
| DPX | distyrene, plasticizer, xylene |
| EDTA | ethylenediaminetetraacetic acid |
| FDC | Franz diffusion cells |
| HEPES | n-[2hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] |
| HRP | horseradish peroxidase |
| HSV | *Herpes simplex* virus |
| IHC | immunohistochemistry |
| LOX | lipoxygenase |
| PRE | pomegranate rind extract |
| RIPA | radioimmunoprecipitation assay buffer |
| TFF | tannin-free fraction |
| TPT | total pomegranate tannins |
| WB | Western blotting |
| 96WP | 96 well (microtitre) plate |

1. **Introduction**

There is a clinical need for more and improved anti-inflammatory products. Many disease states are associated with inflammation. This is a natural response by the body's defences and is essential in the tissue repair process; however, chronic inflammation is associated with pain and discomfort, and has been implicated as a preliminary stage in life-threatening conditions such as cancer and cardiovascular disease [1]. The immunologic basis for skin inflammation diseases such as psoriasis, allergic contact dermatitis and atopic dermatitis, with emphasis on potentially effective targets for novel anti-inflammatory drugs, was recently reviewed [2].

The processes involved in skin inflammation occur in the viable epidermis, which is largely composed of functional keratinocytes. During inflammatory challenge to the skin the keratinocytes respond by releasing cytokines and activating arachidonic acid metabolism along the COX-2 and lipoxygenase (LOX) pathways [3]. COX-2 is also rapidly upregulated upon chemical or mechanical tissue injury, signalling the arachidonic pathway to produce prostaglandin inflammatory mediators. The upregulation of COX-2 and LOX is transient with a short half-life and therefore is useful as a marker to determine the level of inflammation within skin at particular timepoints. Analysis of the level of COX-2 expression in skin *ex vivo* provides a comparative model for the anti-inflammatory anti- (or pro-) inflammatory responses of topically applied xenobiotics, and has been used to study for example n-3 fatty acids, ketoprofen and an extract of *Harpagophytum procumbens* have shown this [4,5].

The pomegranate, fruit of the *Punica granatum L.* tree, has been used since ancient times to treat a wide range of ailments. Pomegranate rind extract (PRE) is

obtained from the pericarp (rind), which contains the highest concentration of phytochemicals, principally polyphenolic flavonols and ellagitannins, including ellagic acid and punicalagin. It has recently been shown that the innate antimicrobial activity of PRE is significantly potentiated by the co-administration of zinc (II) ions with up to 7-log reduction potency observed against *Herpes simplex* virus (HSV) - the causative microorganism of anogenital herpes and oral coldsores [6]. HSV type 1 and 2 lesion eruptions cause a significant level of inflammation to the localised area, resulting in erythema, swelling and pain, particularly in the later stages of infection. Furthermore, other HSV-related conditions, such as herpetic stromal keratitis, are also associated with upregulated COX-2 expression [7].

Although challenging the virus is the primary concern when treating such conditions, a substantial vital load reduction would take time to feedback into a reduction of inflammation. Thus there would be distinct advantages if a medication could simultaneously address this inflammation-mediated discomfort and appearance directly. In terms of a pomegranate-based product, the notion is underpinned by a growing body of work including two reviews that illustrate the beneficial effects of pomegranate extracts as potential inflammation treatments [8,9]. The positive effect of the consumption of pomegranate extracts and juice has been demonstrated in relation to inflammation in the gastrointestinal tract, where ellagic acid was stated as the responsible agent [10,11]. Polyphenolics of differing structure regulate inflammation-involved pathways in different ways while attenuating colitis [12]. The flavanol quercetin, was found to suppress the expression of COX-2 mRNA in the pouch exudates cells of a rat paw, indicating that the anti-inflammatory action of quercetin may partly due to suppressing the up-regulation of COX-2 [13];

pomegranate aqueous extract was found to inhibit COX expression in mice [14] and reduce inflammatory processes in patients with type-2 diabetes [15].

The purpose of this study was to probe the effect of topically applied PRE, purified tannins (TPT) and $ZnSO_4$ on COX-2 expression following their individual application, or in combination, on porcine skin *ex vivo*. Porcine ears have been used extensively as an acceptable model for human skin in the percutaneous penetration of xenobiotics [16,17]. Anti-inflammatory effects were assessed using Western blotting and immunocytochemistry to determine the modulation of endogenous epidermal COX-2 levels. Skin penetration depth profiles were also determined for punicalagin and Zn (II) by tape stripping.

## 2. Materials and methods

### *2.1. Materials*

Pomegranates, of Spanish origin, were obtained from a local supermarket. Radioimmunoprecipitation assay buffer (RIPA buffer, comprised of 50 mM tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride serine protease inhibitor), Hanks balanced salt solution (HBSS), gentamycin sulphate, Ponseau S, PBS + 0.05% TWEEN, aprotinin, leupeptin, anti-actin antibody, sodium azide, SDS 10%, paraffin wax pellets, phosphate buffer saline pH 7.4 (PBS), methyl green, distyrene/plasticizer/xylene (DPX mountant) and λ-carrageenan were all purchased from Sigma-Aldrich, Poole, UK. Zinc sulphate ($ZnSO_4$), potassium hydrogen phthalate, trifluoroacetic acid and HPLC-grade solvents were obtained from Fisher

Scientific (Loughborough, England). Primary COX-2 antibody (#4842) was purchased from Cell Signalling Technology (Boston, USA). Horseradish peroxidase (HRP)-labelled anti-rabbit polymer, DAB chromagen plus substrate was purchased from Dako UK (Ely, England). Western blocking reagent was from Roche Diagnostics, GmbH (Mannheim, Germany). Rainbow Marker (10-250 Kd), anti-mouse HRP, (HRP)-linked antibody were from Amersham Biosciences Ltd (Amersham, UK). Dura substrate was from Perbio, Cramlington, UK, and 3,3'-Diaminobenzidine (DAB) chromagen-AB substrate was from Abcam, Cambridge, UK. Freshly excised porcine ears were obtained from a local abattoir prior to steam cleaning and immersed in iced Hanks buffer before being subjected to laboratory experimentation within 1 h. As these were excised from freshly slaughtered pigs their use was not subject to ethical approval.

## 2.2. Preparation of Pomegranate Rind Extract (PRE)

The rinds of 6 pomegranates (of Spanish origin) were excised by scalpel and cut into thin strips, blended in deionised $H_2O$ (25% w/v) and boiled for approximately 10 min. The crude solution was then transferred into 50 mL screw-capped tubes and centrifuged at 10,400g at 4°C for 30 min using a Beckman Coulter Avanti J25 Ultracentrifuge and vacuum filtered through a Whatman 0.45 μm nylon membrane filter (Fisher Scientific, Loughborough, UK) before being freeze-dried and stored at -20°C until required. The PRE was reconstituted in pH 4.5 phthalate buffer by adding 2 mg to 10 mL buffer and sonicated for 10 min at 50-60 Hz until fully dissolved - this particular buffer and pH were used as it provided the optimum activity against HSV [6]. Punicalagin was analysed by HPLC (section 2.8.) and consisted of 20% w/w of

the freeze-dried PRE. The chemical structure and a chromatogram of punicalagin showing its composition of two anomers is shown in Figure 1.

*2.3. Preparation of Total Pomegranate Tannins (TPT) and Tannin-Free Fraction (TFF)*

Total pomegranate tannins (TPT) is the purified form of PRE which has been stripped of its non-polyphenolic constituents and was prepared by column chromatography using a glass column slurry packed with 75g of Amberlite XAD-16 resin in $H_2O$ [18]. The resin was washed with 300 mL methanol then 100 mL $H_2O$ and left for 12 h to equilibrate. A mass of 5 g PRE (dry weight) was loaded onto the column - the optimal loading volume $40 \pm 5$ mL per 75g of preconditioned XAD-16 resin per column and was eluted with 300 mL of $H_2O$ until the pale yellow tannin-free fraction (TFF) was fully removed. The remaining tannins were eluted with 100 mL MeOH to yield a dark red TPT solution, which was dried under vacuum to yield TPT (freeze dried yield 1.3 g, 26%) and TFF (freeze dried yield 3.4 g, 68%). It was determined that this fraction contained 1.09 g of punicalagin (84% of the TPT fraction), and 0.009 g of ellagic acid (0.69%). These findings are in accordance with the literature which states that punicalagin was equivalent to 80-85% and ellagic acid 1.3% of the total pomegranate tannin content [18]. HPLC revealed no evidence of tannins present within the TFF fraction (not shown).

*2.4. Ex Vivo Skin Preparation and Topical Delivery*

The ears were excised immediately post mortem and immersed in iced Hanks buffer, before arriving in the laboratory within 1 hour. The ears were subsequently cleaned, hairs trimmed and dorsal full thickness skin excised by blunt dissection, whilst being continually bathed in Hanks buffer. The skin sheets were then cut into 2 $cm^2$ sections and used immediately.

Topical delivery was performed using all-glass Franz diffusion cells (FDC) [19] which had a nominal diffusional area of 0.88 $cm^2$ and receptor phase volume of 3 mL. The skin was mounted stratum corneum uppermost on the lightly pre-greased flanges of the receptor and the donor chamber was placed on top of the membrane and clamped into position. A micro-stirrer bar was added to the receptor compartment, filled with degassed Hanks buffer as the receptor phase, and the sampling arm capped. The complete cells were placed on a multiple stirrer plate in a thermostatically controlled water bath set at 37°C for 15 min to allow the temperature to reach equilibrium prior to dosing (Table 1). A dose of 1 mL of the solutions was applied, with the intention of showing a maximal effect, rather than simulate an in-use scenario. At predetermined times the cells were dismantled and the skin membranes retained, prior to biological analysis or tape stripping.

*2.5. Western Blotting (WB) Analysis of COX-2*

The test solutions of PRE, PRE + $ZnSO_4$, $ZnSO_4$, TPT, TFF and control (phthalate buffer pH 4.5) were applied to *ex vivo* full thickness porcine skin within a Franz diffusion cell for 6 h. SDS PAGE and Western blotting was then carried out for COX-2, as an inflammatory marker, and β-Actin, as the protein loading control.

The relative levels of expression of the inflammatory enzyme COX-2 were quantified following liberation from the tissue by skin lysis [20]. To prepare the skin

lysates, the diffusional areas of the skin were carefully excised by scalpel, cut into small pieces and added to 1 mL RIPA lysis buffer; the protease inhibitors were added immediately prior to homogenising for 1 min using a probe instrument (Silverson, Chesham, UK). Lysates were incubated for 15 min on ice then pelleted by centrifugation at 1400 $g$ for 2 x 15 min at 4°C and the supernatant stored at -20°C. Methods for protein estimation and denaturation, polyacrylamide gel electrophoresis and Western blot analysis, are all described in detail elsewhere, and the method has previously been validated by showing the effect of known anti-inflammatory drugs as positive control: ketoprofen, ibuprofen and betamethasone dipropionate [4,5,21].

*2.6. Immunohistochemical (IHC) Analysis of COX-2*

To supplement the Western blotting data the relative levels of COX-2 expression in skin were visualised using immunohistochemistry (IHC) which refers to the process of detecting antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues [22]. This process then allows qualitative or semi quantitative analysis of anti-inflammatory effect by comparing the COX-2 levels as indicated by colour intensity, whereby greater intensity indicates higher analyte concentrate and lower intensity indicates lower analyte concentration. The method as used in these laboratories is explained in detail elsewhere [4].

*2.7. Penetration Depth Profiling*

Tape stripping is widely used for the comparative estimation of the skin penetration of xenobiotics from topically applied formulations [19,23,24]. At 24 h

post-dosing, the skin was recovered from the FDC and excess formulation carefully removed using cotton buds. Regular Sellotape pieces, approx. 2.5 cm in length, were then applied over the diffused area and light pressure applied, before removal with forceps. The first two strips were discarded as they typically contain formulation lodged within the crevices of the skin surface, rather than being deposited within the tissue. Further tape pieces were then applied to the same area using uniform pressure and removed with uniform force, before being placed in screw capped glass vials and extracted with 4 mL methanol using an electronic rocker for 24 h. The strips were then removed and the methanol evaporated to dryness before the residue was reconstituted with 1mL deionised $H_2O$. This was then centrifuged at 15000 $g$ for 15 min and the supernatant transferred to an autosampler vial, before being analysed for punicalagin by HPLC and zinc by ICPMS.

*2.8. Analysis of Punicalagin by Reverse-Phase High Performance Liquid Chromatography (HPLC)*

In this work punicalagin was used as marker for PRE – punicalagin (Figure 1) is the tannin in highest concentration in PRE and has previously been shown to exert the greatest effect on *Herpes simplex* virus in combination with zinc (II) [6]. The analysis was performed using an Agilent series 1100 HPLC system fitted with a Phenomenex Gemini NX C18 110Å 250 x 2.6 mm column. Gradient elution was used, involving A = methanol with 0.1% trifluoroacetic acid (TFA) and B = deionised $H_2O$ with 0.1% TFA: 0 min A 5% B 95%, 15 min A 20% B 80%, 30 min A 60% B 40%, 40 min A 60% B 40%. Injection volume was 20 □L and detection was by UV at 258nm. Punicalagin naturally occurs as a pair of anomeric isomers, α and β, in the

ratio of 1:2 (Figure 1). Aqueous solutions of punicalagin standard were analysed over a range of concentrations and the resulting calibration curves of the α and β anomers were used to determine total punicalagin levels by summation of the areas of the two corresponding peaks in the test sample chromatograms.

*2.9. Analysis of Zinc by Inductively Coupled Plasma Mass Spectrometry (ICPMS)*

The levels of zinc permeating the skin were determined by ICPMS analysis [25] using a Thermo Elemental X Series 2 ICP-MS system equipped with a Plasma Screen. Calibration was carried out using synthetic standard solutions prepared from single element stock standards and analysis was performed by injecting each solution using $^{66}Zn$ as the analytical mass. Periodic checks for accuracy were performed by analysis of a solution of the international rock standard JB1a as an unknown - this standard was prepared by digesting a sample in $HF/HNO_3$ and then $HNO_3$. The resulting concentration data were plotted as cumulative permeation versus time (n=3 ± SD).

*2.10. Data Analysis*

The Western blot bands were scanned and analysed using a densitometer (GS-690 Imaging Densitometer, Bio-Rad Laboratories Ltd, Herts, UK) equipped with Alpha DigiDoc software. The data obtained was recorded and analysed using Excel 2007 (Microsoft office, Redmond, WA). Each experiment was performed in triplicate for each sample and the result expressed as the mean ± SD Statistical analysis was

carried out with InStat for Macintosh, version 3.0 (GraphPad Software Inc, San Diego, CA).

## 3. Results and Discussion

### 3.1. Western Blotting of Skin Lysate for COX-2

COX-2 is an inducible enzyme that is generally expressed only in cells where prostaglandins are upregulated, e.g., during inflammation. It is short-lived with a lifespan of 1-2 min at $V_{max}$ and is permanently inactivated after converting a few hundred arachidonic acid molecules [26]. Freshly excised porcine skin contains elevated levels of the COX-2 under viable conditions and it will continue to be produced until an anti-inflammatory agent is present that can block the enzyme or interfere with another part of the inflammatory pathway, or the tissue loses viability. Viability can be maintained in FDCs using certain media, in this current work we used HEPES buffered Hanks' balanced salt solution (27). This technique has previously been used in our laboratories to examine the potential anti-inflammatory effects of a wide range of compounds applied to skin [4,5,21]. In doing so, such work has the added advantage of simultaneously verifying whether or not a permeant compound penetrates the skin in sufficient levels to enable it to act upon cellular COX-2. Clearly, no step in the arachidonic acid pathway can be modulated if insufficient compound penetrates to the dividing cells of the viable epidermis.

Figure 2 shows the bands produced by WB analysis for COX-2 expression at ~ 72 kDa and the protein loading control of β-actin at ~42 kDa. Densitometric measurements of the bands for COX-2 were normalised using β-actin; levels of COX-2 expression in the control were arbitrarily assigned a value of 100% and test

solutions were shown as a percentage of the control and graphically displayed as a histogram. The application of PRE and PRE + ZnSO$_4$ both led to the statistically significant ($p>0.01$) reduction of COX-2 expression by $66.5 \pm 3.6$ % and $64.5 \pm 5.1$ % respectively. Application of ZnSO$_4$ had no significant ($p>0.05$) effect on the level of COX-2 expression. The reduction of COX-2 by PRE was similar to that reported previously [28] where it was shown a COX-2 reduction of 79% in colon cancer cells, and the lack of COX-2 reduction by the application of ZnSO$_4$ at this concentration has also been previously shown [29]. Previous work using this *ex vivo* skin model confirmed anti-inflammatory activity from known anti-inflammatory agents included as positive control [4,5,21].

No reduction in COX-2 expression was observed using TFF, indicating that the components eliciting the major anti-inflammatory effect are the tannins. COX-2 was downregulated following the application of TPT at 40.5%, but this level was significantly higher (by 26%) than the result obtained from PRE, in the presence or absence of ZnSO$_4$. The reduction in COX-2 expression is greatest when PRE remained as a whole extract, with TPT (with its high punicalagin content) exhibiting anti-inflammatory activity, although to a lesser extent - this is again similar to reported findings [18].

## 3.2. Immunohistochemical (IHC) Staining of Ex Vivo Skin for COX-2

To verify the results observed from the WB experiments, samples were subject to visual analysis using IHC. As expected, high levels of COX-2 was clearly observed in the untreated control skin at 0 h, as shown by the dark brown staining (Figures 3 & 4), after 6 h the degree of staining, and thus the inflammation, is

sustained and that by 24 h the COX-2 level increased (Figure 3). Previous work has

shown that after 24 h necrosis occurs in such skin samples, making the determination

of further time points impossible [4]. Figure 3 also shows that after 6 h treatment with

PRE there was a clear reduction in COX-2, and a similar reduction was observed after

the application of PRE + $ZnSO_4$ combined. The level of COX-2 expression, after

application of PRE alone and in combination with $ZnSO_4$, was further reduced over

24 h as shown by the lighter staining observed in Figures 3 & 4 at this time point.

Application of $ZnSO_4$ was found to have no effect on the level of COX-2 expression

over the 24 h timescale. IHC analysis thus provided visual qualitative confirmation

that the topical application of PRE (with and without $ZnSO_4$) reduced COX-2

expression in the *ex vivo* skin, which indicates a reduction in the inflammatory

response. This is in agreement with several previous reports. Oral dosing of

pomegranate to mice was reported to inhibit UVB-induced inflammation by

modulating NF-κB and MAPK signalling [30]. Other work reported the pretreatment

of rat primary microglia with punicalagin (5-40 μM) prior to LPS (10 ng $mL^{-1}$)

stimulation which produced a significant ($p < 0.05$) inhibition of TNF-α, IL-6 and

prostaglandin E2 production; protein and mRNA expressions of COX-2 and

microsomal prostaglandin E synthase 1 were also reduced by punicalagin

pretreatment [31].

Results from the current work demonstrate that the topical application of TPT

and particularly PRE to skin affects the arachidonic acid inflammation pathway by

downregulating the expression of COX-2. This suggests the biosynthesis in the viable

epidermis may be blocked by one or more compound [3,15], although other steps

such as DNA interaction or prostaglandin sequestration could feedback to produce a

similar result. Nevertheless, reduction in COX-2 expression was statistically

significant after 6 h and was maintained up to 24 h - as this occurred in skin with an intact stratum corneum this shows that punicalagin and other components of PRE and TPT are able to penetrate the skin despite being large molecules, in agreement with our other work [32]. When PRE and $ZnSO_4$ were applied in combination a statistically similar downregulation of COX-2 was observed when compared to the application of PRE alone, illustrating no modulatory effect of the presence of the zinc (II), and there was no evidence of anti-inflammatory activity following the application of $ZnSO_4$ over this time period, in agreement with other work [33]. Our earlier paper, involved the topical application a PRE and zinc sulphate hydrogel, and showed that anti-inflammatory activity was retained by the permeants in FDC receptor phases that were then used to dose *ex vivo* skin, using the methods described in the current paper [34].

Using a commercial MTS kit, previous work has shown the absence of cytotoxicity of the solutions used in the current work [6]; indeed cytotoxic effects, if they occurred, might be expected to upregulate COX-2 and be observed in the current results [35]. As there was no evidence of this, the conclusion is that the formulations are not cytotoxic at the levels used.

*3.2. Depth profiles of Punicalagin and Zinc*

To further prove that the anti-inflammatory effects observed above were entirely attributable to the penetration of PRE, we next determined the penetration of punicalagin and zinc within the skin. The permeation (i.e. delivery into and out from the membrane) of punicalagin and zinc across skin and mucosal membranes prone to HSV infection has been recently published [34]. Here we were concerned with

establishing the levels localised within skin and that constitute a drug reservoir [19], as this would clearly have a bearing on the ability of the applied dose to modulate skin inflammation processes as observed above. Tape stripping is a crude analytical technique that is widely used for the comparative determination of skin penetration [23,24], although specific mapping of strips to epidermal stratification yields more precision in determining drug localisation. Nevertheless, on a purely relative basis, Figure 5 shows that the penetration of punicalagin was quite constant throughout each layer of the epidermis after 24 h, although higher amounts in the upper layers would typically be observed [19,36]. Here, by far the greatest recovery was from the remaining skin (i.e. remaining epidermis and dermis) and reflects the elevated levels observed in the basal layer by the technique of reverse-tape stripping [34]. The amount of punicalagin recovered in each layer of the epidermis after application of PRE and application of PRE + $ZnSO_4$ was in the region of 0.1- 0.2 nM $cm^{-2}$ and 0.05 - 0.1 nM $cm^{-2}$ respectively. No penetrant peaks were observed for the control, as expected. The level of punicalagin after the application of PRE was $2.39 \pm 0.29$ nM $cm^{-2}$. After application of PRE and $ZnSO_4$ the concentration of punicalagin was somewhat lower at $3.3 \pm 0.47$ nM $cm^{-2}$ ($p < 0.05$). The total recovery of punicalagin after application of PRE and PRE + $ZnSO_4$ from the tape strips and the dermis was not statistically different ($p>0.05$) at $3.58 \pm 0.1$ nM $cm^{-2}$ and $3.91 \pm 0.62$ nM $cm^{-2}$ respectively. These results are consistent with the permeation of punicalagin through the epidermis after application of 40 µL of PRE (1 mg $mL^{-1}$) and PRE (1 mg $mL^{-1}$) + $ZnSO_4$ (1M) [6].

As shown in the skin permeation experiment, punicalagin delivery occurred within the first three to six hours after application [34] and suggests that the punicalagin recovered in each of these layers after 24h application time had reached a

point of equilibrium. Figure 3 shows that a greater amount of punicalagin localised within the remaining skin when PRE was applied in combination with $ZnSO_4$ and that punicalagin had a more even distribution through the stratum corneum and dermis after the application of PRE alone, the reason for the differences is unclear but may involve a complexation or co-permeation mechanism [37].

Figure 6 shows the amount of zinc recovered from the tape stripping of full thickness porcine skin after the topical application of 40 µL PRE (1 mg mL$^{-1}$) + $ZnSO_4$ (1 M), $ZnSO_4$ (1 M) and control (pH 4.5 phthalate buffer) *in vitro*. The control shows that significant endogenous zinc leached from the porcine skin. Zinc was recovered from the porcine skin within each layer of the stratum corneum and within the dermis the total quantity recovered was $68.03 \pm 5.6$ nM cm$^{-2}$ - this value is comparable with that reported previously [38]. The concentration of zinc recovered was greatest and significantly different ($p>0.05$) after the application of $ZnSO_4$ and PRE + $ZnSO_4$, this remained true at all layers of the stratum corneum. The highest levels were again extracted from the remaining skin − remnant epidermis and dermis. This could be due to preferential binding to the basal layer and/or the small size of the zinc (II) atom lead to rapid delivery into the receptor phase.

Overall, dosing of PRE and $ZnSO_4$ to *ex vivo* porcine skin indicates that punicalagin, the major virucidally active polyphenolic tannin within PRE, and zinc penetrate throughout the epidermis, supporting our work in relation to skin permeation [34]. However, the greater amounts of both analytes in the remaining skin may, given the polar nature of the analytes and the vehicles, may also reflect the importance of the appendageal delivery routes, in particular via the sweat pores. These are found over the entire body with a density of 400 glands cm$^{-2}$ and have the purpose of exuding sweat, an aqueous electrolyte solution [39]. Given the clear anti-

inflammatory effects observed, such channels could provide a short-cut by allowing the permeants to bypass the barrier function of the stratum corneum which is primarily lipoidal in nature. This is sometimes referred to as a 'shunt route' [40], and also goes some way to explaining the amounts founds in relation to their physicochemical properties: the molecular weights of punicalagin and zinc (II) are 1084 and 65 respectively, both markedly different to the generally accepted maximum of ~400 [19] and unfavourable fitting to the Potts and Guy algorithm (not shown) [41]. Such may have been facilitated by the infinite dosing of a liquid vehicle, although it should be noted that our previous paper involving a hydrogel formulation [34] also revealed significant COX-2 downregulation in ex vivo skin. Generally, a molar excess of zinc were found in the tape strips relative to punicalagin, reflecting the different loading concentrations.

## 4. Conclusions

The current findings show that topically applied TPT and PRE, with or without zinc (II), exert a significant anti-inflammatory effect on COX-2 expression in *ex vivo* skin. Indirectly, this demonstrates that the anti-inflammatory principles, in particular punicalagin, penetrate the skin and modulate COX-2 expression; penetration into skin was further supported by tape stripping data. Pomegranate extracts are generally safe [42,43] and could therefore represent a novel approach in ameliorating the inflammation and pain associated with a range of skin conditions. When considered alongside the potent virucidal activity [6], there is the basis of a dual action therapeutic system for HSV infections, such as coldsores, anogenital herpes and herpetic stromal keratitis.

**Acknowledgment**

## References

[1] D. Laveti, M. Kumar, R. Hemalatha, R. Sistla, V.G. Naidu, V. Talla, V. Verma, N. Kaur, R. Nagpal, Anti-inflammatory treatments for chronic diseases: a review, Inflamm. Allergy Drug Targets 12 (2013) 349-361.

[2] C. Albanesi, S. Pastore, Pathobiology of chronic inflammatory skin diseases: interplay between keratinocytes and immune cells as a target for anti-inflammatory drugs, Curr Drug Metab. 11 (2010) 210-227.

[3] E. Ricciotti, G. FitzGerald, Prostaglandins and inflammation. Arterioscler. Thromb. Vasc. Biol. 31 (2011) 986–1000.

[4] C.P. Thomas, Z. Davison, C.M. Heard, Probing the skin permeation of fish oil/EPA and ketoprofen-3. Effects on epidermal COX-2 and LOX, Prostag. Leukotr. Ess. 76 (2007) 357-362.

[5] N. Abdelouahab, C.M. Heard, Effect of *Harpagophytum procumbens* and its major iridoid glycosides on epidermal COX-2 in vitro, J. Nat. Prod. 71 (2008) 746-749.

[6] D.M.J. Houston, Towards a nanomedicine based broadspectrum topical virucidal therapeutic system. PhD Thesis, Cardiff University, 2011.

[7] P.S. Biswas, K. Banerjee, B. Kim, P.R. Kinchington, B.T.Rouse, Role of inflammatory cytokine-induced cycloxygenase 2 in the ocular immunopathologic disease herpetic stromal keratitis, J. Virol. 79 (2005) 10589-10600.

[8] E.P. Lansky, R.A. Newman, Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer, J. Ethnopharmacol. 109 (2007) 177–206.

[9] T. Ismail, P. Sestili, S. Akhtar, Pomegranate peel and fruit extracts: A review of potential anti-inflammatory and anti-infective effects, J. Ethnopharmacol. 143 (2012) 397–405.

[10] M. Larrosa, A. González-Sarrías, M.J. Yáñez-Gascón, M.V. Selma, M. Azorín-Ortuño, S. Toti, F. Tomás-Barberán, P. Dolara, J.C. Espín, Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. J. Nutr. Biochem. 21 (2010) 717-725.

[11] E. Colombo, E. Sangiovanni, M. Dell'Agli, A review on the anti-inflammatory activity of pomegranate in the gastrointestinal tract. Evid. Based Complement. Alternat. Med. 2013 Article ID 247145.

[12] H. Kim, N. Banerjee, I. Ivanov, S. Talcott, S. Mertens-Talcott, Comparison of anti-inflammatory mechanisms of mango (*Mangifera indica L.)* and pomegranate (*Punica granatum L.)* in DSS-induced colitis in rats (372.8). FASEB J. 28 (2014) Supplement 372.8.

[13] K. Morikawa, M. Nonaka, M. Narahara, I. Torii, K. Kawaguchi, T. Yoshikawa, Y. Kumazawa, S. Morikawa, Inhibitory effect of quercetin on carrageenan-induced inflammation in rats. Life Sci. 74 (2003) 709–721.

[14] F. Afaq, M. Saleem, C.G. Krueger, J.D. Reed, H. Mukhtar, Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice. Int. J. Cancer 113 (2005) 423–433.

[15] G. Sohrab, J. Nasrollahzadeh, H. Zand, Z. Amiri, M. Tohidi, M. Kimiagar, Effects of pomegranate juice consumption on inflammatory markers in patients with

type 2 diabetes: a randomized, placebo-controlled trial. J. Res. Med. Sci. 19 (2014) 215–220.

[16] I.P. Dick, R.C. Scott, Pig ear skin as an in-vitro model for human skin permeability. J. Pharm. Pharmacol. 44 (1992) 640-645.

[17] A. Summerfield, F. Meurens, M.E. Ricklin, The immunology of the porcine skin and its value as a model for human skin. Mol. Immunol. 66 (2015) 14–21.

[18] N.P. Seeram, L.S. Adams, S.M. Henning, Y. Niu, Y. Zhang, M.G. Nair, D. Heber, In vitro antiproliferative, apoptopic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. Nutr. Biochem. 16 (2005) 360-367.

[19] A.C. Williams, Transdermal and Topical Drug Delivery from Theory to Clinical Practice. Pharmaceutical Press, UK, 2003.

[20] D. Laouini, A. Elkhal, A. Yalcindag, S. Kawamoto, H. Oettgen, R.S. Geha, COX-2 inhibition enhances the TH2 immune response to epicutaneous sensitization. J. Allergy Clin. Immunol. 116 (2005) 390-396.

[21] M.H. Zulfakar, N. Abdelouahab, C.M. Heard, Enhanced topical delivery and ex vivo anti-inflammatory activity from a betamethasone dipropionate formulation containing fish oil. Inflamm. Res. 59 (2010) 23-30.

[22] L.L. de Matos, D.C. Trufelli, M.G.L. de Matos, M.A. da Silva Pinha, Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomark. Insights 5 (2010) 9–20.

[23] J.J. Escobar-Chávez, V. Merino-Sanjuán, M. López-Cervantes, Z. Urban-Morlan, E. Piñón-Segundo, D. Quintanar-Guerrero, A. Ganem-Quintanar, The tape-stripping technique as a method for drug quantification in skin. J. Pharm. Pharm. Sci. 11 (2008) 104-130.

[24] J. Lademann, U. Jacobi, C, Surber, H.J. Weigmann, T.W. Fluhr. The tape stripping procedure – evaluation of some critical parameters. Eur. J. Pharm. Biopharm. 72 (2009) 317–323.

[25] G. Li, J.D. Brockman, S.W. Lin, C.C. Abnet, L.A. Schell, J.D. Robertson, Measurement of the trace elements Cu, Zn, Fe, and Mg and the ultratrace elements Cd, Co, Mn, and Pb in limited quantity human plasma and serum samples by inductively coupled plasma-mass spectrometry. Am. J. Anal. Chem. 3 (2012) 646-650.

[26] N.V. Chandrasekharan, D.L. Simmons, The cyclooxygenases. Genome Biol. 5 (2004) 241.

[27] R.L. Bronaugh, Methods for in vitro skin metabolism studies. In, K.P. Wilhelm, H. Zhai, H.I. Maibach, Dermatotoxicology 8th Edition, Chapter 43. CRC Press, Boca Raton, FL, USA, 2013.

[28] L.S. Adams, N.P. Seeram, B.B. Aggarwal, Y. Takada, D. Sand, D. Heber, Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signalling in colon cancer cells. J. Agric. Food Chem. 54 (2006) 980-985.

[29] M. Roselli, A. Finamore, I. Garaguso, M.S. Britti, E. Mengheri, Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli*. J. Nutr. 133 (2003) 4077-4082.

[30] N Khan, D.N. Syed, H.C. Pal, H. Mukhtar, F. Afaq, Pomegranate fruit extract inhibits UVB-induced inflammation and proliferation by modulating NF-κB and MAPK signaling pathways in mouse skin. Photochem. Photobiol. 88 (2012) 1126-1134.

[31] O.A. Olajide, A. Kumar, R. Velagapudi, U.P. Okorji, B.L. Fiebich, Punicalagin inhibits neuroinflammation in LPS-activated rat primary microglia. Mol. Nutr. Food Res. 58 (2014) 1843-1851.

[32] M.V. Berridge, A.S. Tan, Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT. Arch. Biochem. Biophys. 303 (1993) 474-482.

[33] M.S. Agren, M. Chvapil, L. Franzen, Enhancement of re-epithelialization with topical zinc oxide in porcine partial-thickness wounds. J. Surg. Res. 50 (1991) 101-105.

[34] D.M.J. Houston, J.J. Bugert, S.P. Denyer, C.M. Heard, In vitro penetration and biological activity of punicalagin and zinc (II) across skin and mucous membranes prone to HSV infection. Eur. J. Pharm. Sci. 96 (2017) 99-106.

[35] W. Thomas, Z.K. Ascott, D. Harmey, L.W. Slice, E. Rozengurt, A.J. Lax, Cytotoxic necrotizing factor from Escherichia coli induces RhoA-dependent expression of the cyclooxygenase-2 Gene. Infect Immun. 69 (2001) 6839-6845.

[36] C.P. Thomas, C.M. Heard, Probing the skin permeation of fish oil/EPA and ketoprofen 2. Comparative depth profiling and metabolism of eicosapentaenoic acid. Eur. J. Pharm. Biopharm. 67 (2007) 156-165.

[37] C.M. Heard, Ethanol and other alcohols: Old enhancers, new perspectives. In Dragicevic N, Maibach HI (eds) Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement. Springer, Berlin, Germany. p.p. 151-172, 2015.

[38] A.O. Gamer, E. Leibold, B. Ravenzwaay, The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin. Toxicol. In Vitro 20 (2006) 301-307.

[39] H. Schaefer, T.E. Redelmeier, Skin Barrier Principles of Percutaneous Absorption. Karger, Basel, Switzerland, 1996.

[40] G.L. Flynn, B. Stewart, Percutaneous drug penetration: choosing candidates for transdermal development. Drug Dev. Res. 13 (1988) 169-185.

[41] R. Potts, R. Guy, Predicting skin permeability, Pharm. Res. 9 (1992) 663–669.

[42] C. Patel, P. Dadhaniya, L. Hingorani, M.G. Soni, Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies. Food Chem. Toxicol. 46 (2008) 2728-2735.

[43] A. Vidal, A. Fallarero, B.R. Peña, M.E. Medina, B. Gra, F. Rivera, Y. Gutierrez, P.M. Vuorela, Studies on the toxicity of Punica granatum L. (Punicaceae) whole fruit extracts. J Ethnopharmacol. 89 (2003) 295-300.

**Legends to figures**

Figure 1 Chemical structure and high performance liquid chromatography chromatogram of punicalagin, showing the 1:2 proportions of $\alpha$ and $\beta$ anomers.

Figure 2 Analysis of cyclooxygenase-2 (COX-2) protein expression by Western blotting. Full thickness porcine skin was treated with topical $ZnSO_4$ (1 M), pomegranate rind extract (PRE) (1 mg mL$^{-1}$), PRE (1mg mL$^{-1}$) + $ZnSO_4$ (1 M) and phthalate buffer as a control for 6 h, protein was extracted and 30 $\mu$g was loaded and separated via SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The histogram represents COX-2 (72kDa) levels normalised against $\beta$-actin (42 kDa). Levels in control were arbitrarily assigned a value of 100% (n=3 $\pm$ SD).

Figure 3 Immunohistochemical staining of porcine skin for cyclooxygenase-2 (COX-2) at 0h, 6h, and 24h after the topical application of control (phthalate buffer pH 4.5) and pomegranate rind extract (1 mg mL$^{-1}$) (40x magnification). Darker shading is due to higher COX-2 presence, lighter shading is due to lower COX-2 presence (representative of 3 determinations).

Figure 4 Immunohistochemical staining of porcine skin for cyclooxygenase-2 (COX-2) at 0h, 6h, and 24h after the topical application of PRE (1 mg mL$^{-1}$) + $ZnSO_4$ (1 M) and $ZnSO_4$ (1 M) (40x magnification). Darker shading is

due to higher COX-2 presence, lighter shading is due to lower COX-2 presence.

Fig 5 Depth profile of punicalagin ($\alpha + \beta$) in porcine skin after application of pomegranate rind extract (PRE) (green), PRE + $ZnSO_4$ (red) and control (blue) (n=3 ± SD).

Fig 6 Depth profile of zinc recovered from the tape stripping of full thickness porcine skin after 24h application of pomegranate rind extract (1 mg mL$^{-1}$) + $ZnSO_4$ (1 M), $ZnSO_4$ (1M) and control (n=3 ± SD).
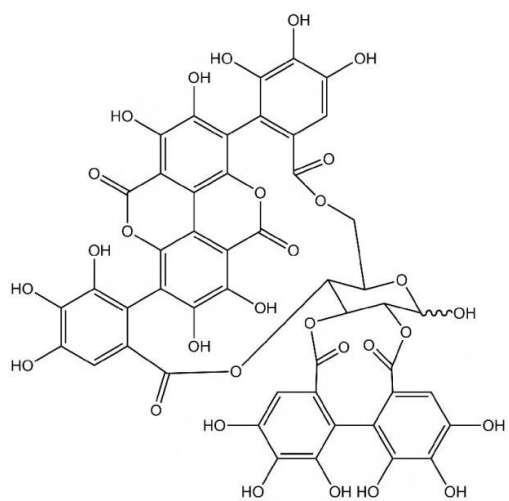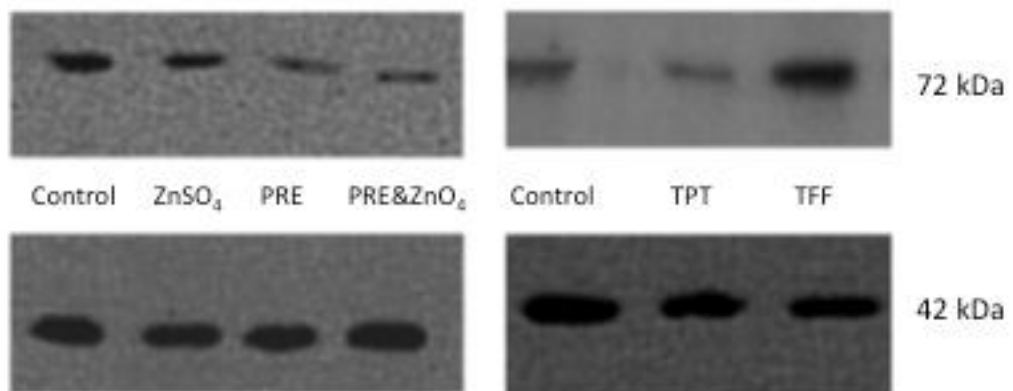
Fig 1

Fig 2

| Control 0h | Control 6h | Control 24h |
| PRE 0h | PRE 6h | PRE 24h |
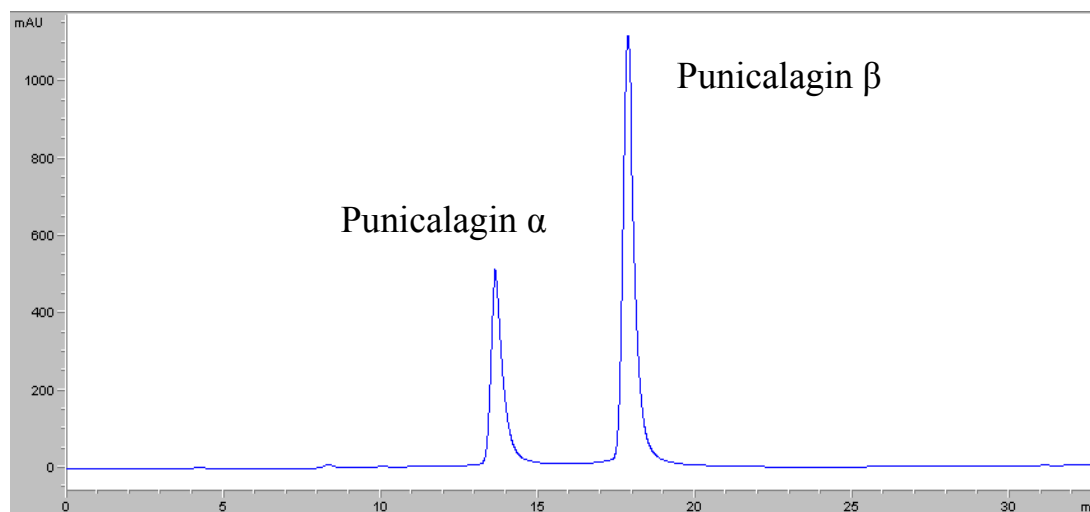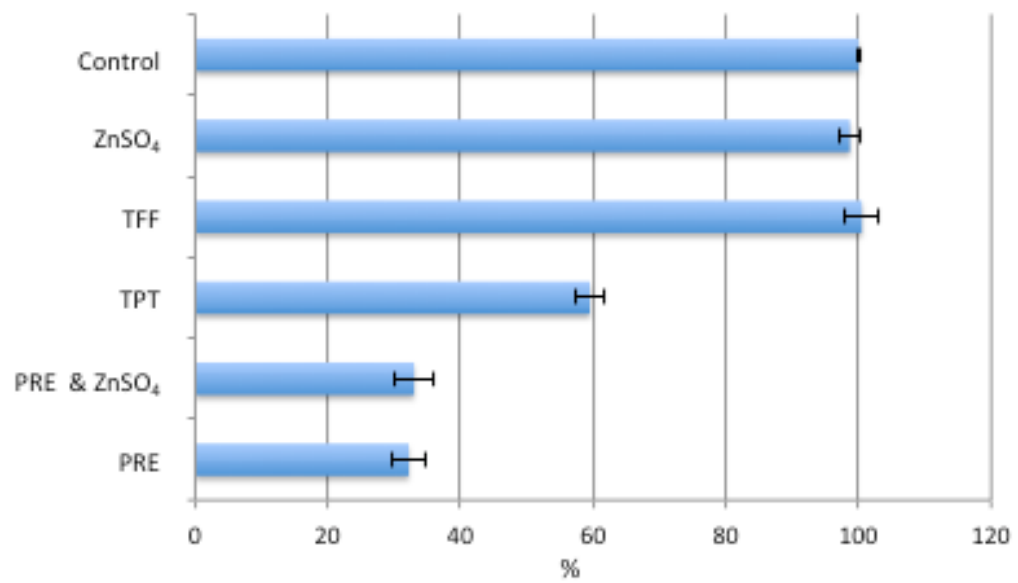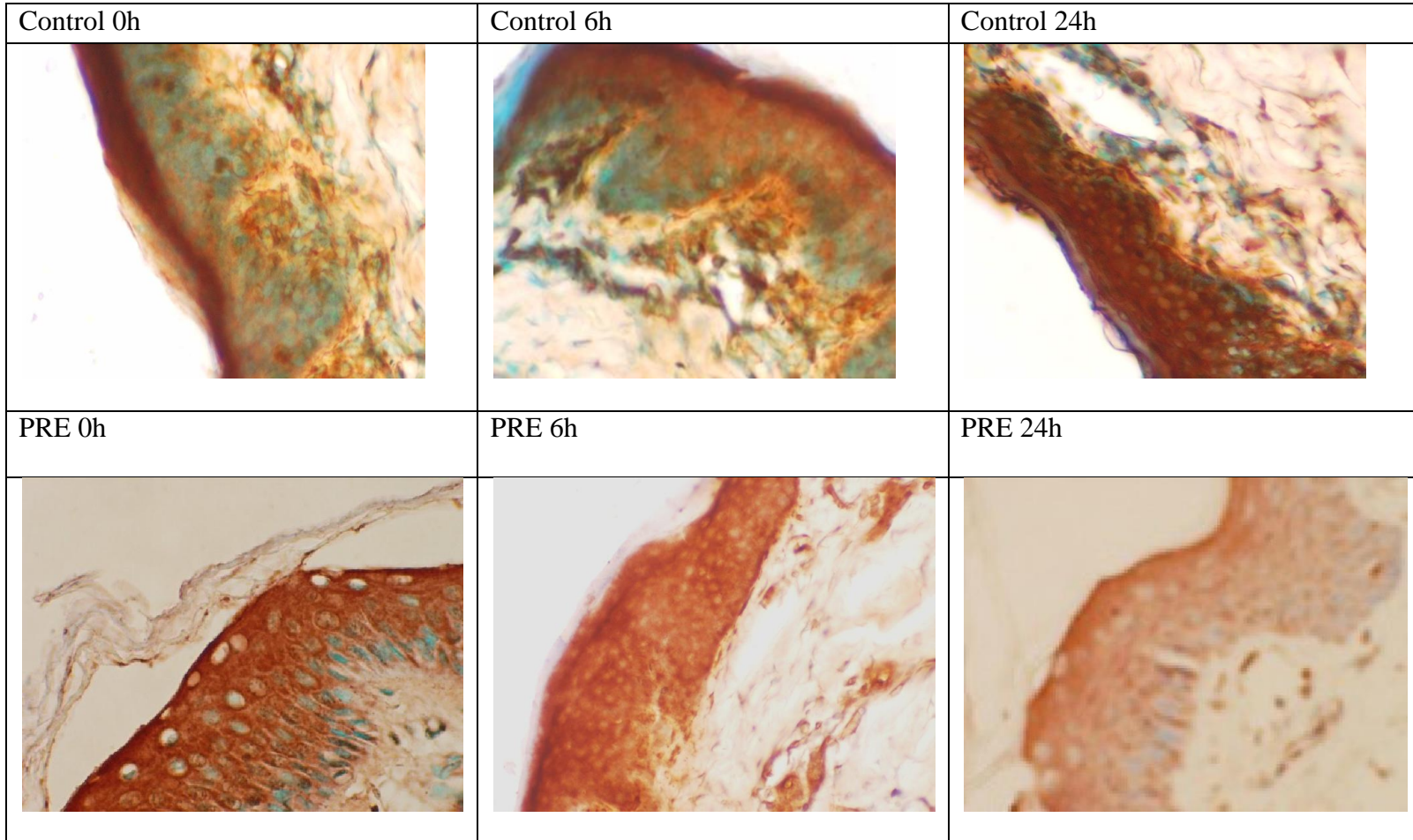
Figure 3

Figure 4
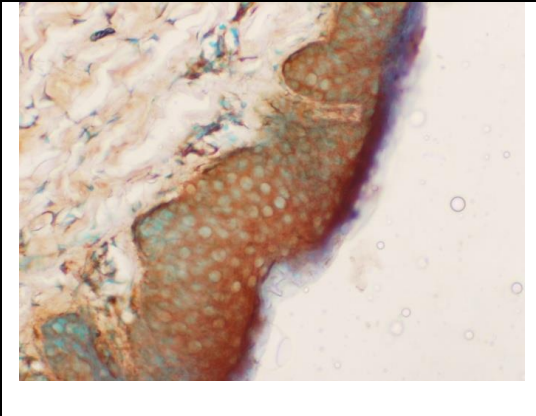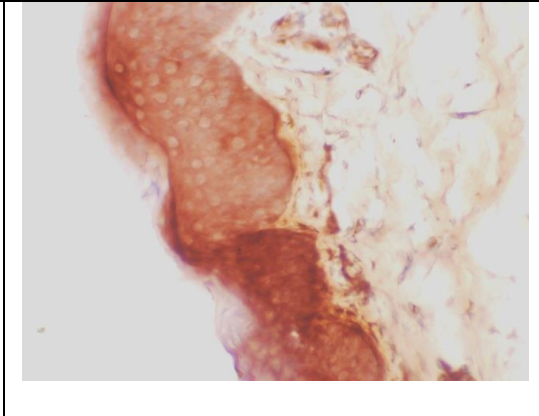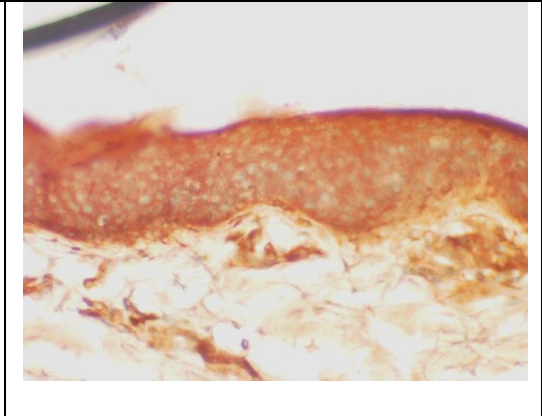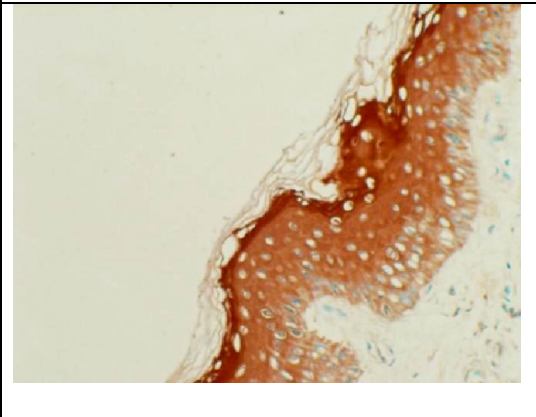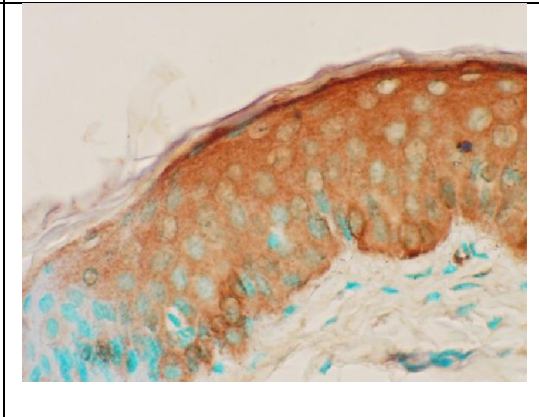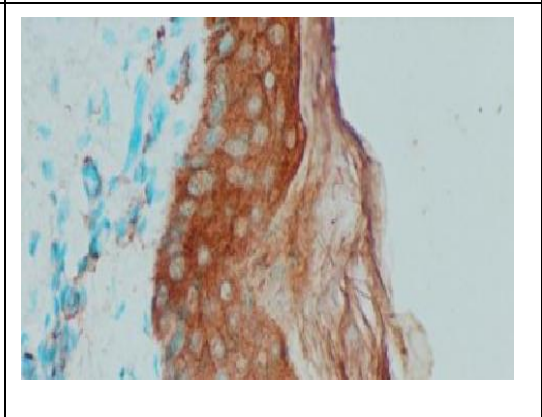
| PRE + ZnSO$_4$ 0h | PRE + ZnSO$_4$ 6h | PRE + ZnSO$_4$ 24h |
|---|---|---|
|  |  |  |
| ZnSO$_4$ 0h | ZnSO$_4$ 6h | ZnSO$_4$ 24h |
|  |  |  |

Fig 5
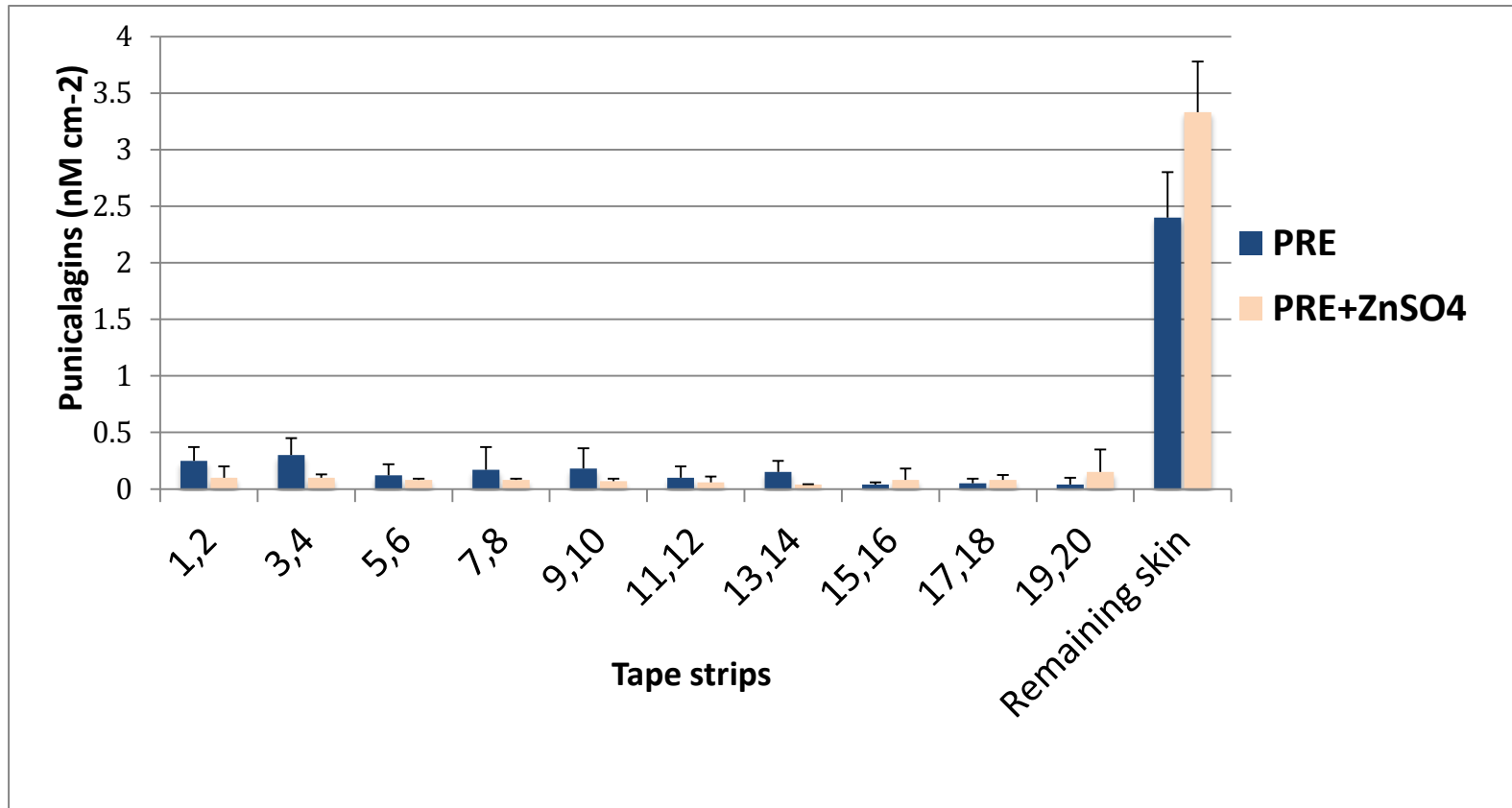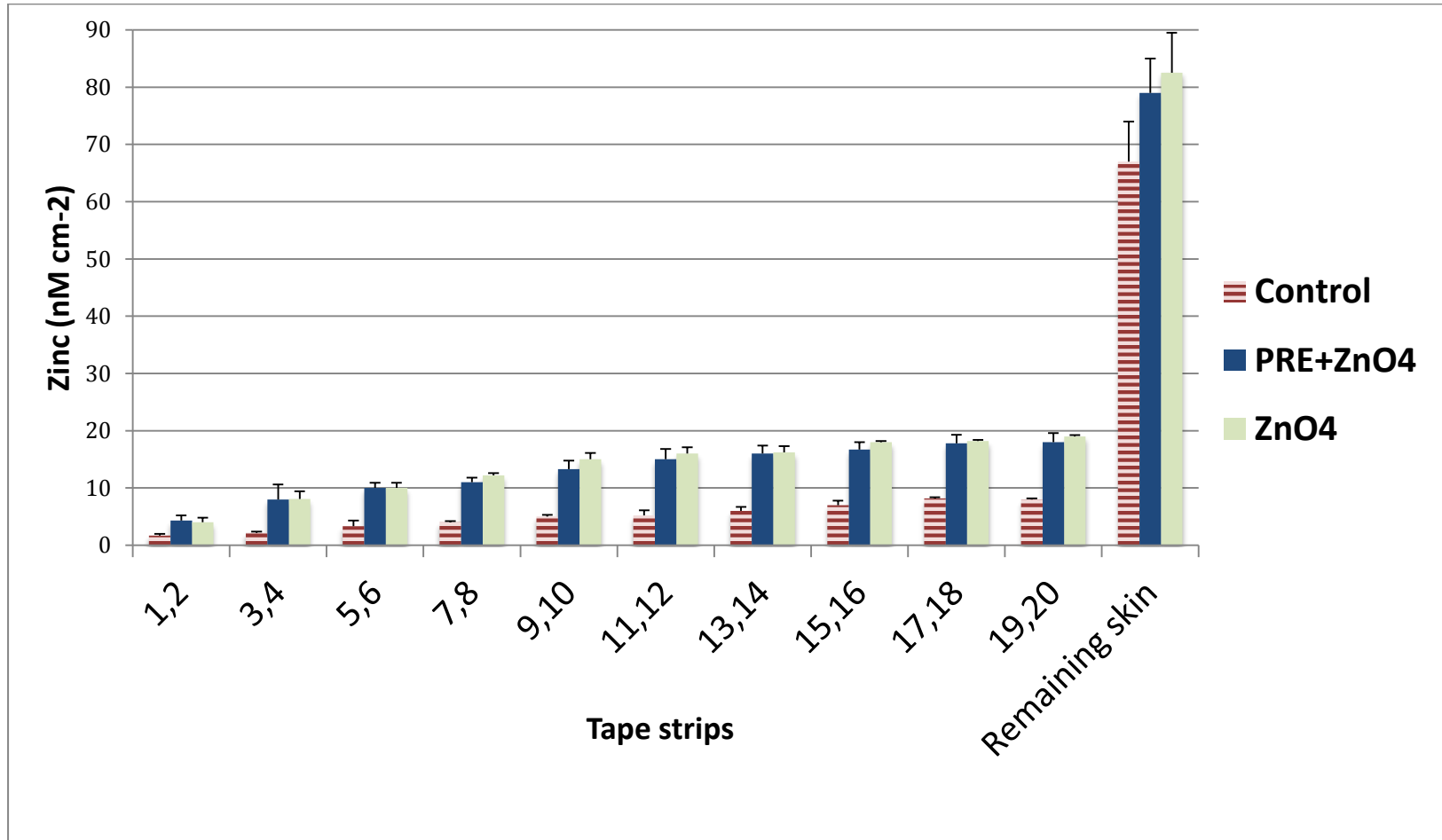
Fig 6

| Test solution | Vehicle | Molecular biology technique |
|---|---|---|
| PRE | 0.1 mg mL$^{-1}$ in phthalate buffer pH 4.5 | Immunohistochemistry Western Blotting |
| ZnSO$_4$ | 0.1 M in phthalate buffer pH 4.5 | Immunohistochemistry Western Blotting |
| PRE + ZnSO$_4$ | PRE 0.1 mg mL$^{-1}$, ZnSO$_4$ 0.1 M, phthalate buffer pH 4.5 | Immunohistochemistry Western Blotting |
| Control | Phthalate buffer pH 4.5 | Immunohistochemistry Western blotting |
| TPT | 0.1 mg mL$^{-1}$ in phthalate buffer pH 4.5 | Western blotting |
| TFF | 0.1 mg mL$^{-1}$ in phthalate buffer pH 4.5 | Western blotting |

Table 1 Test solutions and molecular biology techniques employed in the assessment of anti-inflammatory activity in *ex vivo* skin (PRE = pomegranate rind extract, TPT = total pomegranate tannins, TFF = tannin-free fraction). 1mL of test solution dosed in each case, n=3.