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In vitro and *ex vivo* examination of topical Pomiferin treatments

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ABSTRACT

Pomiferin is a unique, prenylated isoflavonoid that can be isolated and purified from the fruits of *Maclura pomifera* (Osage Orange). The molecule typically is isolated with a small amount of a molecule called Osajin which is structurally similar to Pomiferin but lacks an aromatic hydroxyl group. As a consequence, Osajin has been shown to be a less effective antioxidant than Pomiferin. *In vitro* studies on Normal Human Dermal Fibroblasts demonstrate that Pomiferin is a potent extracellular matrix protein stimulant, showing increases in collagen, elastin and fibrillin expression comparable or superior to equivalent concentrations of retinol. *Ex vivo* hair follicle assays demonstrate comparable effects on expression of collagen and elastin at Pomiferin concentrations in the range of 0.05–5 ppm. Taken together, the results from the two assays conducted on different models indicate that Pomiferin may be a very interesting ingredient for topical skin and scalp treatments where modulation of the expression of extracellular matrix proteins is important.

1. Introduction

Pomiferin is a prenylated isoflavonoid originally isolated and identified from the fruits of Osage Orange (*Maclura pomifera*) by Wolfrom et al., in 1939 [1,2], Fig. 1A. The crystal structure of Pomiferin, however, was not fully resolved until 2003 by Marek et al. [3]. Pomiferin is typically isolated with another structurally similar prenylated isoflavonoid called Osajin [4], Fig. 1B. Studies examining the antioxidant efficiency of these two molecules have demonstrated that Pomiferin is a stronger antioxidant than Osajin [5–7]. This is most likely due to the molecular arrangement of Pomiferin that places two hydroxyl groups on adjacent aromatic carbon atoms in the B-ring. The molecular structure of adjacent conjugated hydroxyl groups is common in strong antioxidants such as EGCG and Vitamin C.

Studies on the influence of Pomiferin on various human cell lines have been reported. In particular, some studies have focused on the influence of Pomiferin on cancer cell lines [8,9]. Svasti et al., examined the effects of Pomiferin on a cholangiocarcinoma cell line, suggesting that the molecule has the ability to kill such cells *in vitro*. Likewise, Yang et al., examined the effects of Pomiferin on breast epithelial cancer cells suggesting that the molecule has selectivity towards such cancer cells, again in *in vitro* assays. Moon et al., examined the effects of Pomiferin as a histone deacetylase inhibitor on various cancer cell lines *in vitro* noting that Pomiferin had the ability to inhibit histone deacetylase activity which may correlate with inhibition of tumor growth [10]. To date, there have been no clinical trials with Pomiferin as a potential cancer treatment. In addition, Pomiferin has been suggested to have antimicrobial efficacy against a number of pathogenic microorganisms including *Staphylococcus aureus*, *Mycobacterium smegmatis*. *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus gallinarum* [11].

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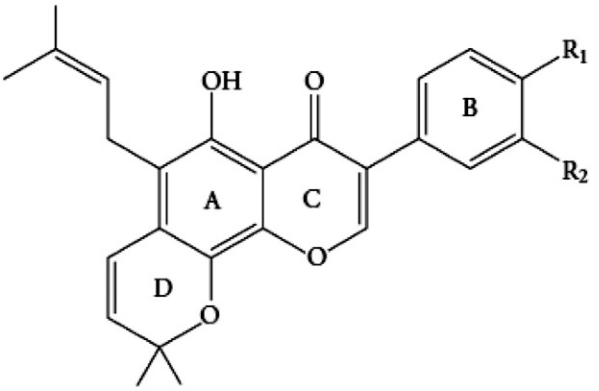

Fig. 1. A–B, Pomiferin, R_1, R_2 = OH: Osajin, R_1 = OH, R_2 = H.

Studies intended to examine the effects of Pomiferin on skin cells have not been reported. Recently, Gruber et al., reported on studies in which normal human dermal fibroblasts and normal human epidermal keratinocyte were exposed to Pomiferin (and Osajin) and gene arrays were run comparing these molecules against a number of other popular skin antioxidants including, for example, EGCG, rosavin, genistein *etc.* [12]. However, these studies did not provide any specific information on how Pomiferin might actually influence expression of skin extracellular matrix proteins like collagen and elastin.

This paper will report on efforts to examine the effect of Pomiferin on normal human dermal fibroblasts and normal human epidermal keratinocytes to see if the molecule might have benefits as a topical skin care active. This paper will report for the first time on studies examining the influence of Pomiferin to stimulate extracellular matrix protein expression, in particular, type 1A1 collagen, elastin and fibrillin expression in fibroblasts. Using an *ex vivo* hair follicle model, the effects of Pomiferin on collagen expression noted in the fibroblasts *in vitro* assay concurred with findings of the *ex vivo* hair follicles. Taken together, the two assays suggest that Pomiferin may be a very interesting ingredient for topical skin and scalp treatments.

2. Materials

2.1. Pomiferin and retinol

Pomiferin was purchased from Gaia Chemical (Gaylordsville, CT) and was approximately 90% pure with the remaining 10% being essentially Osajin. The material was used in the assays without further purification. Commercially available retinol (>95%) was purchased from Sigma-Aldrich (Milwaukee, WI) and was used without further purification.

3. *In vitro* tissue equivalent experimental methods

3.1. Preparation of fibroblasts

Human dermal fibroblasts were obtained from Cascade Biologics and seeded into the individual wells of a 24-well plate in 0.5 ml of Fibroblast Growth Media (FGM: DMEM supplemented with 2% fetal bovine serum (FBS), 50 μg/ml gentamicin, 250 μg/ml amphotericin B, 2 ng/ml human basic fibroblast growth factor, 10 μg/ml human Insulin) and incubated overnight at 37 ± 2 °C and 5 ± 1% CO_2. On the following day the media were removed *via* aspiration to eliminate any non-adherent cells and replaced with 0.5 ml of fresh FGM. The cells were grown until confluent, with a media change every 48 to 72 h. Upon reaching confluency the cells were treated for 24 h with DMEM supplemented with 1.5% FBS to wash out any effects from the growth factors included in the normal culture media. After this 24-hour wash out period the cells were treated with the test materials at the specified concentrations dissolved in DMEM with 1.5% FBS. Retinol was used as a positive control in all the *in vitro* studies. Untreated cells (negative controls) received DMEM with 1.5% FBS. The cells were incubated for 48 h and at the end of the incubation period cell culture medium was collected and either stored frozen (−75 °C) or assayed immediately.

3.2. MTT assay

After the 2-day incubation, the cell culture medium was removed (see above) and the fibroblasts were washed twice with PBS to remove any remaining test material. After the final wash, 500 μl of DMEM supplemented with 0.5 mg/ml MTT was added to each well and the cells were incubated for 1 h at 37 ± 2 °C and 5 ± 1% CO_2. After the incubation, the DMEM/MTT solution was removed and the cells were washed again once with PBS and then 1 ml of isopropyl alcohol was added to the well to extract the purple formazin crystals. Two hundred microliters of the isopropyl extracts was transferred to a 96-well plate and the plate was read at 540 nm using isopropyl alcohol as a blank.

3.3. Procollagen ELISA

Media samples were assayed for type I collagen production using a Type I C-Peptide ELISA kit from Takara Bio Inc. per the manufacturer's instructions.

3.4. Elastin ELISA

Soluble α-elastin from Sigma Chemical was dissolved in 0.1 M sodium carbonate (pH 9.0) at a concentration of 1.25 μg/ml. 150 μl of this solution was then applied to the wells of a 96-well maxisorp Nunc plate and the plate was incubated overnight at 4 °C. On the following day the wells were saturated with PBS containing 0.25% BSA and 0.05% Tween 20. The plate was then incubated with this blocking solution for 1 h at 37 °C and then washed two times with PBS containing 0.05% Tween 20.

A set of α-elastin standards was generated ranging from 0 to 100 ng/ml (prepared in DMEM + 1.5% FBS). 180 μl of either standard or spent media sample from the fibroblasts undergoing the various treatments was then transferred to a 650 μl microcentrifuge tube. An anti-elastin antibody solution was prepared (the antibody was diluted 1:100 in PBS containing 0.25% BSA and 0.05% Tween 20) and 20 μl of the solution was added to the tube. The tubes were then incubated overnight at 4 ± 2 °C. On the following day, 150 μl was transferred from each tube to the 96-well elastin ELISA plate, and the plate was incubated for 1 h at room temperature. The plate was then washed 3 times with PBS containing 0.05% Tween 20.

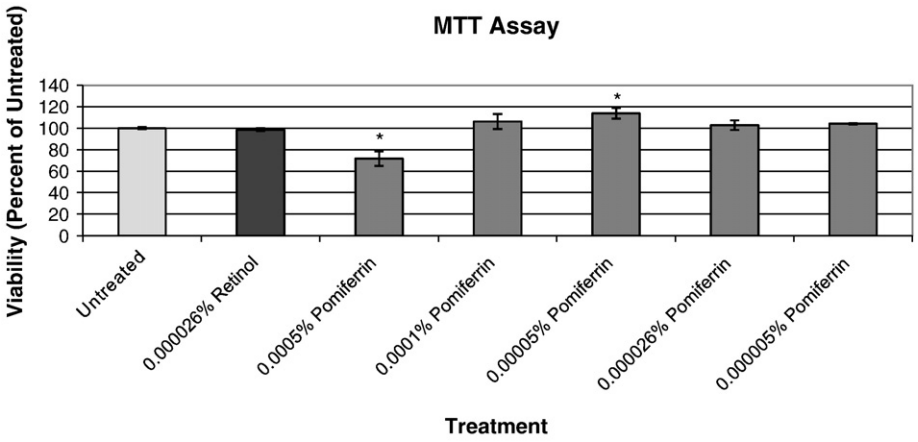

Fig. 2. Results of fibroblast viability assays.

After washing, 200 μl of a solution containing a peroxidase linked secondary antibody diluted in PBS containing 0.25% BSA and 0.05% Tween 20 was added, and the plate was incubated for 1 h at room temperature. After washing the plate three times as described above, 200 μl of a substrate solution was added and the plate was incubated for 10 to 30 min in the dark at room temperature. After this final incubation the plate was read at 460 nm using a plate reader.

3.5. Fibrillin assay

A PVDF membrane was prewet in methanol, equilibrated with TBS (TBS: 20 mM Tris, pH 7.5, 150 mM NaCl) and assembled into the Bio-Dot microfiltration apparatus. After assembly, 100 μl of TBS was added to the wells in the Bio-Dot and the vacuum was applied to ensure that there was an adequate flow through all of the wells. Next, 250 μl of each spent media sample from the fibroblasts undergoing the various treatments was assigned a well in the apparatus and the sample was applied to the appropriate well. After all of the samples had been added, a vacuum was applied to the apparatus to draw the fluid of the samples through the membrane, leaving the protein adhered to the membrane. TBS was added to wells not assigned a sample to ensure that the membrane did not dry out during the procedure. At the end of the blotting procedure the membrane was removed from the Bio-Dot apparatus, washed in TBS for 5–10 min and then placed into blocking solution (TBS with 1% non-fat milk powder) and allowed to incubate for at least 1 h at room temperature on a rocking platform.

After blocking, the membrane was transferred to 20 ml of TBST (TBS with 0.1% Tween-20) and 0.1% non-fat powdered milk with an appropriate dilution of antibody and allowed to incubate overnight at 4 °C on a rocking platform. After this incubation the membrane was washed 3 times (1 for 15 min and 2× for 5 min) in TBST. The secondary antibody (conjugated with a fluorophore) was then incubated with the membrane in 15 ml of TBST with 0.1% non-fat powdered milk for 1 h at room temperature and then washed 3 times with TBS (1× 15 min, 2× for 5 min).

After the final wash, the membrane was placed into a BioRad Molecular Imager FX and scanned using an excitation laser and

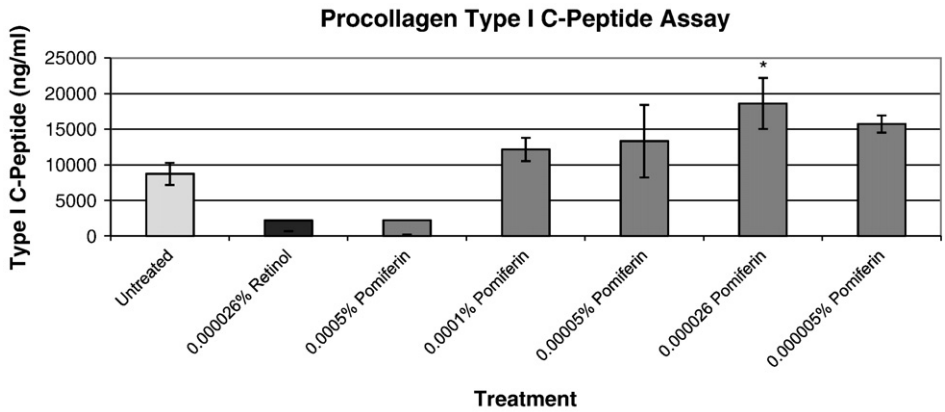

Fig. 3. Results of Type 1A1 Procollagen assay.

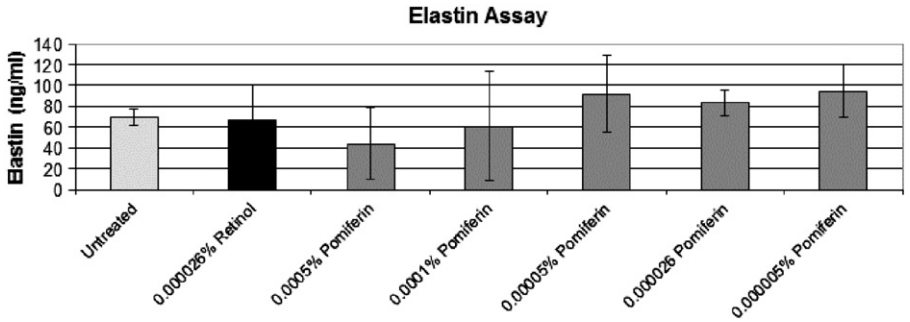

Fig. 4. Results of elastin assay.

emission filter combination appropriate for the fluorophore. Images produced by the scanner were then analyzed using ImageJ image analysis software.

3.6. Calculations

The mean MTT absorbance value for the negative control cells was calculated and used to represent 100% cell viability. The individual MTT values from the cells undergoing the various treatments were then divided by the mean value for the negative control cells and expressed as a percent to determine the change in cell viability caused by each treatment.

For the collagen and elastin ELISA assays, to quantify the amount of each substance present in the spent media samples, a standard curve was generated using known concentrations of each substance and the equation for the line which best fit these standard curve was determined using a regression analysis. For the fibrillin assay, mean fluorescence intensity scores were used for comparison purposes. For all of the assays, mean values were compared using a one way ANOVA. In the results section, statistically significant differences ($P < 0.05$) between a specific treatment and the untreated group are designated with an asterisk.

4. *Ex vivo* hair follicle growth assay

4.1. Isolation of human Anagen IV hair follicles from culture [13]

Normal human scalp tissue (58 y, Female with brown hair) was sourced from elective plastic surgery. Scalp was cleaned in wash solution and carefully sliced immediately below the dermal/fat junction. Anagen VI hair follicles were isolated under a dissecting microscope and placed in Williams E Medium (WEM; Sigma) containing 2× antibiotic/antifungals, 2 mM Glutamax (Invitrogen, UK), 10 μg/ml Insulin and 10 ng/ml Hydrocortisone. After isolation hair follicles were inspected and any with signs of damage or catagen/telogen morphology were discarded. Intact hair follicles were placed singly in a well of a 48-well culture plate ensuring they were completely submerged in 0.3 ml WEM. Hair follicles were placed in a humidified tissue culture incubator at 37 °C containing 5%

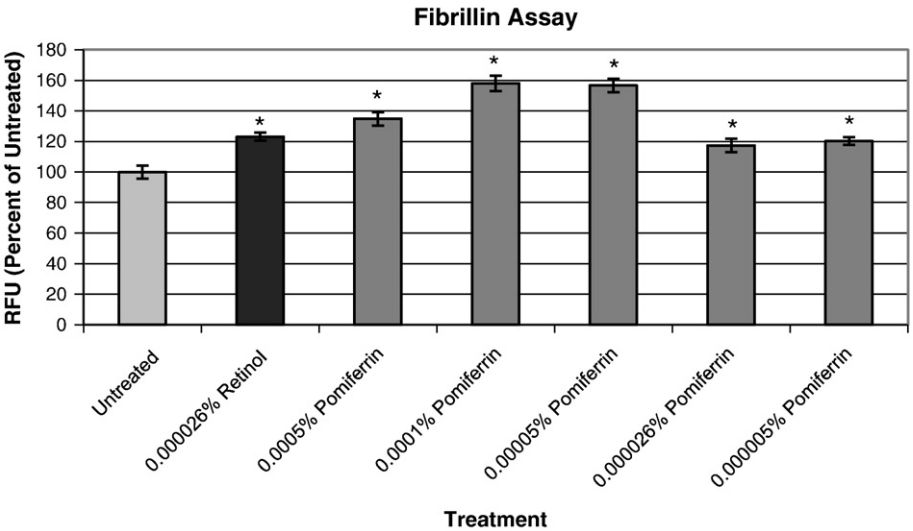

Fig. 5. Results of fibrillin assay.

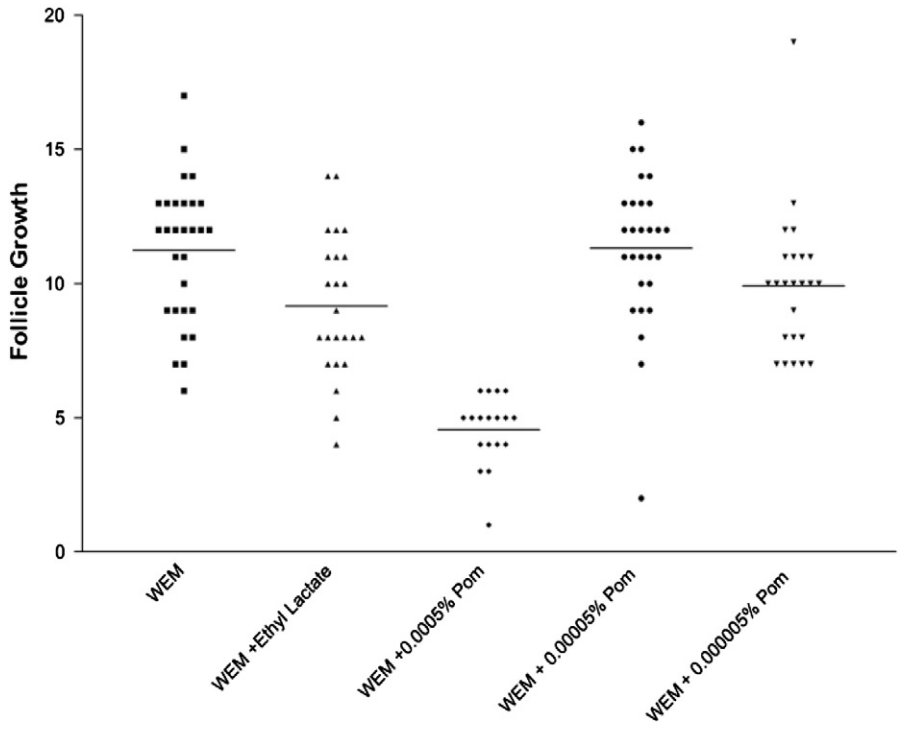

Fig. 6. Measurement of hair elongation for various follicle treatments.

CO2 overnight to check for normal growth. Any hair follicles that did not show overnight growth were discarded.

4.2. Pomiferin treatments

A working stock of 0.5% Pomiferin dissolved in ethyl lactate was diluted in WEM to final test concentrations of 0.0005%, 0.00005% and 0.000005%. Three replicates of 10 follicles for each treatment were incubated in 3 test concentrations of Pomiferin in addition to ethyl lactate vehicle controls (0.1%) and WEM alone. Hair follicles were incubated for 6 days and photographed on day 0 prior to the start of the experiment and at days 2, 4 and 6 (immediately prior to cryopreservation). Growth was assessed by measuring elongation of the hair fiber from the 'shoulder' which demarcated new growth since isolation. Media were replenished on days 2 and 4. Hair follicles were collected for analysis by snap freezing in a small amount of OCT freezing medium. OCT embedded hair follicles were stored at −80 °C until cryosectioning.

4.3. Immunocytochemistry

For immunocytochemistry 10 μm sections were cut and mounted on charged slides (Leica, UK). Sections were air-dried at RT then fixed in ice-cold acetone for 10 min at −20 °C. After equilibrating to RT slides were rinsed in PBS. Non-specific antibody binding was blocked by incubating in 10% donkey serum (Sigma-Aldrich, UK) diluted in PBS for at least 30 min. Serum was poured off and followed by incubation in primary antibody (Tropoelastin (1:100, ab-21605), Elastin (1:100, ab-23747) and Collagen-1 (1:100 and 1:500, ab-34710); Abcam, UK) diluted in PBS containing 1% donkey serum overnight in a humidified slide chamber at 4 °C. Excess primary antibody was rinsed by washing in PBS for 3 × 10 min. Hair follicles were incubated in donkey anti-rabbit Alexa-488 conjugated secondary antibody (Invitrogen Molecular Probes, UK) for 1 h at RT then rinsed in PBS for 4 × 10 min. Slides were mounted for confocal microscopy under sealed coverslips in fluorescent mounting medium containing DAPI nuclear stain (VectorLabs, UK). Images were collected using the 365 nm (UV) and 488 nm (FITC) channels on a Zeiss LSM confocal microscope by sequential line scanning. Images were processed using the LSM confocal image browser software (Zeiss, UK) and ImageJ (freeware).

4.4. Statistical analysis

Hair fiber elongation data were analyzed for statistical significance using one-way ANOVA with Bonferroni post *T*-test comparison (PRISM software).

5. Results and discussion

5.1. In vitro fibroblasts assays

Results of the cytotoxicity of Pomiferin on Normal Human Dermal Fibroblasts using MTT assay procedures are shown below (Fig. 2). Pomiferin is well tolerated by the cells at concentrations below 0.0005%. It has similar cytotoxicity to retinol at comparable concentrations of 0.000026%.

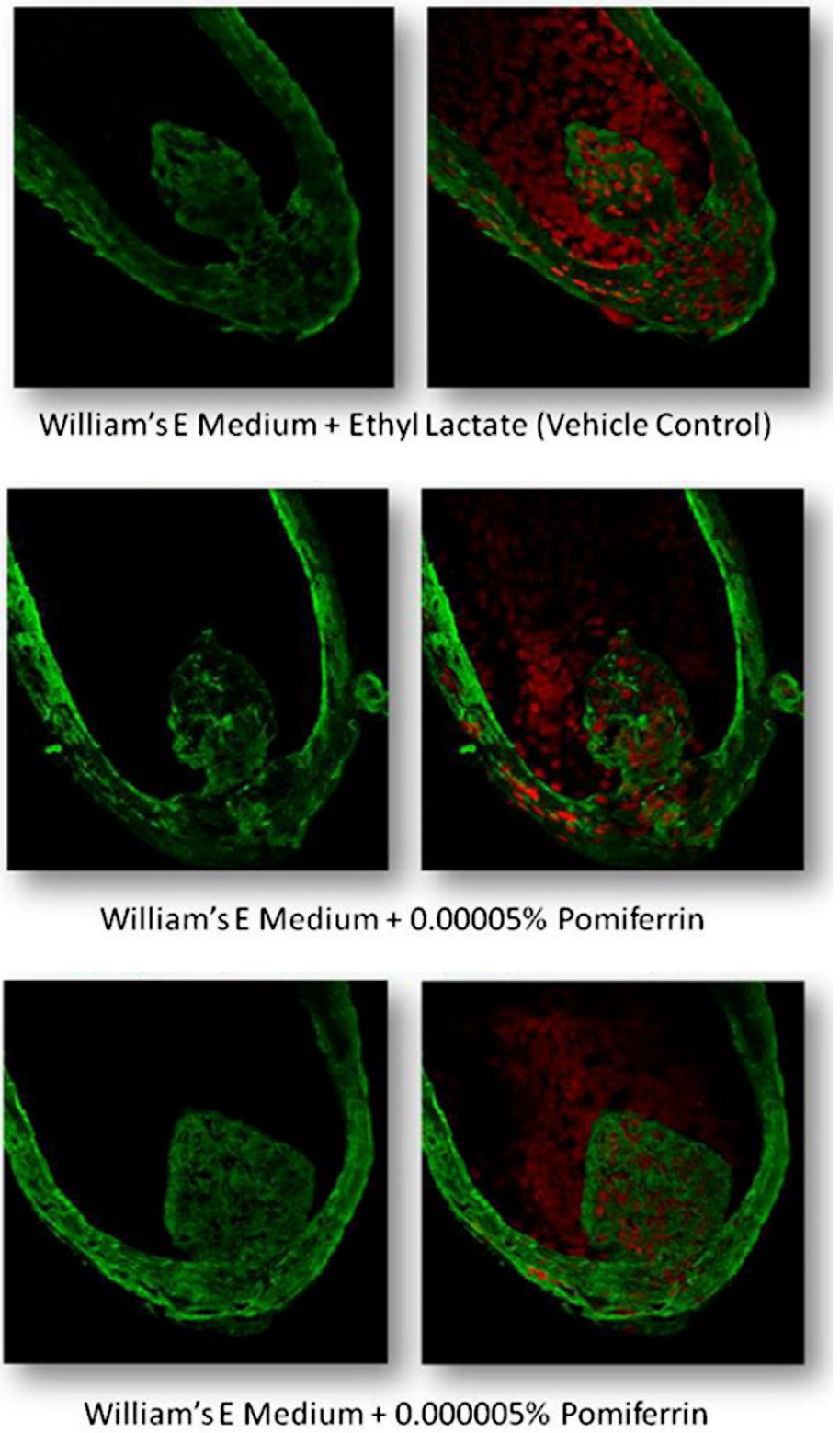

Fig. 7. Photomicrographs of hair follicle histology after treatment with media and ethyl lactate (top) and media, ethyl lactate solvent and Pomiferin (at two concentrations) and immunoprobed for collagen (green). Red indicates nuclei (TOTO-3).

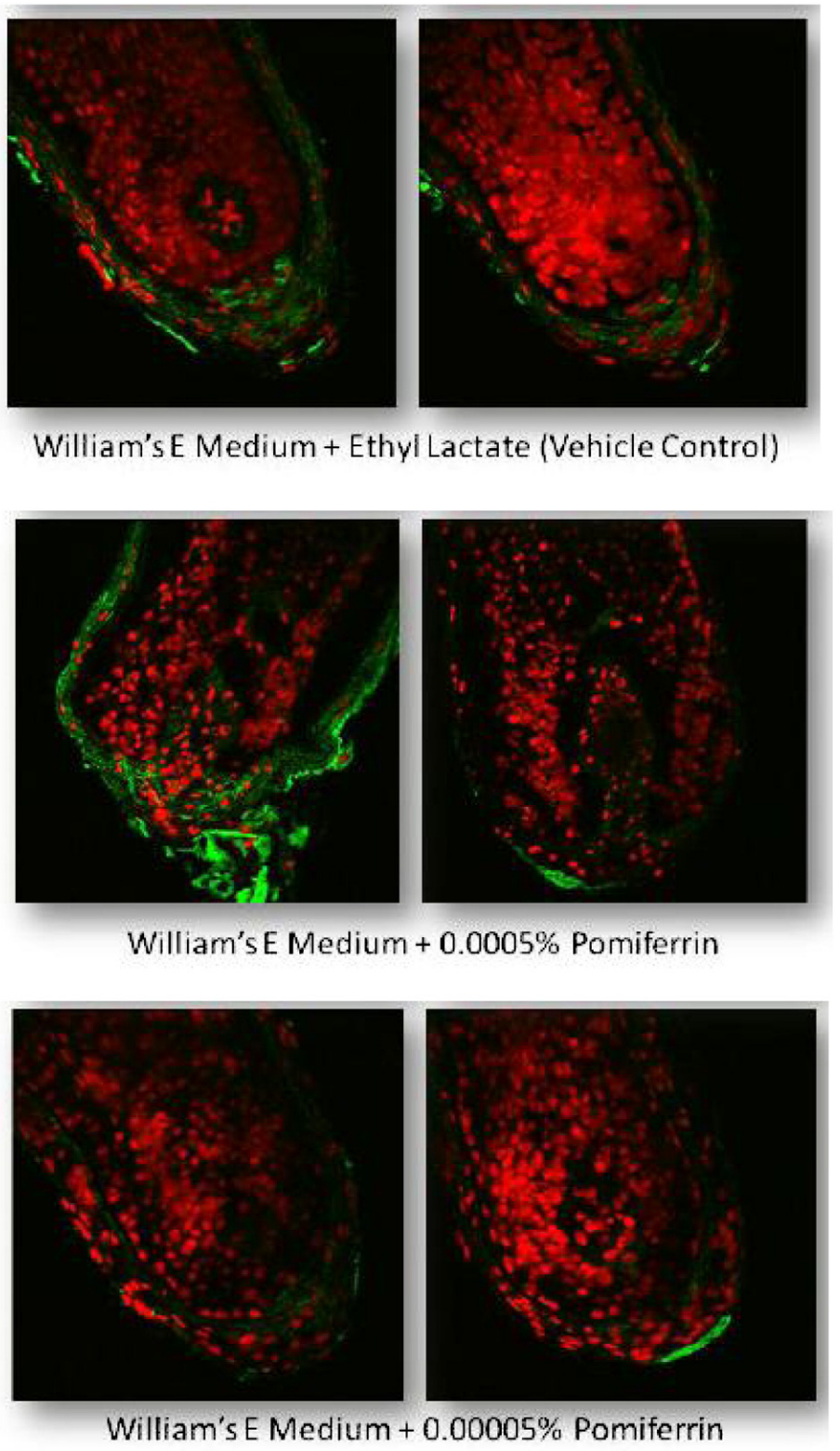

Fig. 8. Photomicrographs of hair follicle histology after treatment with media and ethyl lactate (top) and media, ethyl lactate solvent and Pomiferin (at two concentrations) and immunoprobed for elastin (green). Red indicates nuclei (TOTO-3).

Results of the Type 1A1 Procollagen assay are shown below (Fig. 3). Pomiferin had a significant impact on Type 1A1 Procollagen expression in dermal fibroblasts. The results indicate a significant effect of retinol at the 0.000026% equivalent treatment levels. While both Pomiferin and retinol demonstrated low cell toxicity at 0.000026%, the results of the retinol treatments indicate a possible cytotoxic effect as collagen expression dropped below untreated levels. This did not happen for Pomiferin until concentrations approaching cytoxicity at 0.0005%.

Results of the elastin assay are shown below (Fig. 4). Results from the elastin assay show a comparable effect of treatment with Pomiferin and retinol at 0.000026%. Both molecules stimulated elastin expression in fibroblasts.

Results of the fibrillin protein assay are shown below (Fig. 5). Pomiferin treatment increased fibrillin protein expression in fibroblasts, and was equivalent to retinol at the 0.000026% treatment level.

5.2. Ex vivo hair follicle growth assays

Results of the influence of Pomiferin on hair elongation are shown (Fig. 6). At concentrations shown to be cytotoxic to fibroblasts *in vitro* (0.0005%), Pomiferin also showed a suppression of hair fiber elongation as might be anticipated. However, compared against the media and solvent (ethyl lactate) alone which showed a slight suppression of elongation, Pomiferin at 0.00005% demonstrated a possible ability to elongate *ex vivo* hair fibers although the increase in length did not exceed that seen when hair follicles were treated with medium — without ethyl lactate. Statistical analysis ($P < 0.05$) demonstrated a significant increase in hair fiber length when treated with 0.00005% Pomiferin compared to treatment with media/ethyl lactate-media (Fig. 6).

Histological examination of hair follicles comparing media/ethyl lactate-treated follicles against media/ethyl lactate/Pomiferin-treated hair follicles demonstrates a significant increase in collagen expression in the hair follicles as shown below (Fig. 7). Histological examination of treated hair follicles using collagen-specific immunochemical staining indicates an apparent increase in collagen expression around the hair follicle dermal sheath as indicated by a brighter green fluorescence for the Pomiferin-treated follicles *versus* the hair follicles treated with media and solvent alone at both Pomiferin concentrations. Expression of collagen in the Pomiferin-treated follicles appears stronger at 0.00005% *versus* 0.000005% showing effects comparable to collagen expression in dermal fibroblasts (Fig. 3). Results from the two separate assays correlate well, and indicate that Pomiferin can have a positive effect on collagen expression both *in vitro* and *ex vivo* at concentrations in the range of 5.0–0.05 ppm.

Results of elastin expression in the hair follicles are shown below (Fig. 8). Results of elastin immunochemical staining indicate that Pomiferin also can stimulate elastin expression around the hair follicle at the 0.0005% level (near fibroblast cytotoxic levels) but that it drops off at 0.00005%.

Pomiferin has been shown to be a potent upregulator of extracellular matrix proteins both *in vitro* on dermal fibroblasts and *ex vivo* on hair follicles. The results on dermal fibroblasts indicate that the protein expression is equal to or superior to retinol at comparable concentrations. This suggests that highly purified Pomiferin might be an interesting ingredient for potential skin and scalp care topical treatments where extracellular matrix protein expression is strategically important.

Conflict of interest

The authors report no conflict of interest.

References

[1] Wolfrom ML, Benton FL, Gregory AS, Hess WW, Mahan JE, Morgan PW. J Am Chem Soc 1939;61:2832–6.
[2] Wolfrom ML, Harris WD, Johnson GF, Mahan JE, Moffett SM, Wildi B. J Am Chem Soc 1946;68:406–18.
[3] Marek J, Vesela D, Margita L, Zemlicka M. Acta Crystallogr C 2003;C59:127–8.
[4] Liskova M, Marek J, Jankovska, Sukupova L, Zemlicka M, Vanco J. Acta Crystallogr E 2005;E61:1848–50.
[5] Tsao R, Yang R, Young JC. J Agric Food Chem 2003;51:6445–51.
[6] Vesela D, Kubinova R, Muselik J, Zemlicka M, Suchy V. Fitoterapia 2004;75:209–11.
[7] Diopan V, Babula P, Shestivska V, Adam V, Zemlicka M, Dvorska M, et al. J Pharm Biomed Anal 2008;48:127–33.
[8] Svasti J, Srisomsap C, Subhasitanont P, Keeratichamroen S, Chokchaichamnankit D, Ngiwsara L, Chimnoi N, Pisutjaroenpong S, Techasakul S, Chen ST 2005;5:4504–4509.
[9] Yang R, Hanwell H, Zhang J, Tsao R, Meckling KA. J Agric Food Chem 2011;59:13328–36.
[10] Son IH, Chung IM, Lee SI, Yang HD, Moon HI. Bioorg Med Chem Lett 2007;17:4753–5.
[11] Mahmoud ZF. J Med Plant Res 1981;42:299–301.
[12] Gruber JV, Holtz R. Mediators Inflamm 2010;2010:1–10.
[13] Tobin DJ. *Ex vivo* organ culture of human hair follicles: amodel epithelial–neuroectodermal–mesenchymal interaction system. Methods Mol Biol 2011;695:213–27.