

Effects of pomegranate flower complex on the skin

Today's understanding of skin ageing describes two processes – intrinsic and extrinsic skin ageing. In both cases there is an imbalance between collagen breakdown and collagen synthesis which, in the long term favours collagen breakdown.

Dermal fibroblasts play a significant role in these processes and produce both the components of the extracellular matrix (ECM) and the enzymes responsible for its breakdown and restructuring.¹ The main components of the extracellular matrix are collagen and elastin fibres. Among other things they provide structure, softness and elasticity to the skin and absorb mechanical stresses.¹ Matrix metalloproteinases (MMP) possess endopeptidase activity and are also responsible for the breakdown and modelling of the ECM. There are more than 20 different known types of MMP.² For the investigations into MMPs in skin-ageing processes, MMP-1, MMP-3 and MMP-9 are of interest because they induce the breakdown of collagen.

Intrinsic or chronological skin ageing is based on genetically determined loss in the skin's ability to regenerate. Literature discusses three mechanisms responsible for this, continuous metabolic stress-induced reactive oxygen species (ROS), telomere shortening and changes in hormone balance.^{5,8} The number of dermal fibroblasts drops, collagen breakdown increases and at the same time collagen synthesis decreases. Unlike extrinsic skin

ABSTRACT

The balance between collagen breakdown and collagen synthesis plays a crucial role in the extrinsic and intrinsic process of skin ageing. Environmental factors acting on the skin and genetically-determined processes cause an increase in collagen breakdown and at the same time a reduction in the synthesis of new collagen, which results in the increased appearance of wrinkles. One of the enzymes responsible for the breakdown of collagen is matrix metalloproteinase 1 (MMP-1). It has been demonstrated that an extract of pomegranate flowers reduces MMP-1 mRNA gene expression in UVA irradiated

and non-irradiated primary human dermal fibroblasts. Due to this characteristic, the pomegranate flower extract (PGFE) was combined with sodium ascorbyl phosphate (SAP) into a single product (PGFE/SAP). SAP, a derivative of vitamin C, is a well-known antioxidant and collagen booster. The combined product made of PGFE and SAP (PGFE/SAP) was successfully tested for its safety in use both *in vitro* and *in vivo*. Results showed that PGFE/SAP promoted collagen synthesis, reduced the breakdown of collagen and measurably and visibly reduced the depth of facial wrinkles caused by skin ageing.

ageing, the thickness of the skin is reduced and small, fine lines are formed.^{5,8}

Skin ageing caused by external influences is called extrinsic skin ageing. Extrinsic factors such as radiation, physical and mental stress, alcohol and nicotine, environmental toxins or poor nutrition lead to greater MMP production while at the same time reducing collagen synthesis.^{4,5} Among the ageing factors named, UV irradiation plays a major role. Photoageing, as it is known, describes the process of skin ageing caused by UV irradiation. UVB irradiation with a wavelength of 315 nm to 280 nm acts on the epidermis and damages the DNA of keratinocytes

and melanocytes.⁵ The longer-wave UVA irradiation (380 nm to 315 nm) also damages the epidermis but also penetrates into the dermis beneath. Here it acts on fibroblasts disturbing the synthesis of collagen and encouraging its breakdown.⁵ In addition, ROS are generated which are also evoked by the extrinsic ageing factors mentioned above.^{4,7,8} A thickening of the skin, even a leathery appearance, count among the signs of extrinsic skin ageing together with pigmentation spots and the formation of deep wrinkles.⁴ In particular areas of the skin affected are those which are continually exposed to the sun such as the face, the lower part of the neck/upper chest, the nape of the neck, hands and forearms. Wrinkles caused by solar irradiation and facial expressions develop particularly on the forehead (worry and frown lines), around the eyes (crow's feet) and around the mouth.

The common factors in both types of ageing are the effects of ROS, and an increase in collagen breakdown coupled with a reduction of collagen synthesis. These common factors are possible starting points for modern anti-ageing strategies. Free radicals should be neutralised by topical or oral application of antioxidants. MMPs should be inhibited or their expression suppressed and collagen synthesis boosted.

Table 1: Composition of the cream formulation with 1% product content.

Component	Portion [%]	INCI CFTA
Water	71.48	Water
Glycerin	3.00	Glycerin
Citric acid 50%	0.02	Water (and) Citric Acid
Tego Care 450 ¹	3.00	Polyglyceryl-3 Methylglucose Distearate
Tego Alkanol 6855 ¹	2.50	Cetearyl Alcohol
Myritol 318 ²	13.00	Caprylic/Capric Triglycerides
Jjoba oil (cold processed)	6.00	Simmondsia Chinensis (Jojoba) Seed Oil
PGFE/SAP ³	1.00	Maltodextrin (and) Punica Granatum Flower Extract (and) Sodium Ascorbyl Phosphate
Preservative	qs	
Suppliers: 1 Evonik 2 Cognis 3 Cosmtochem		

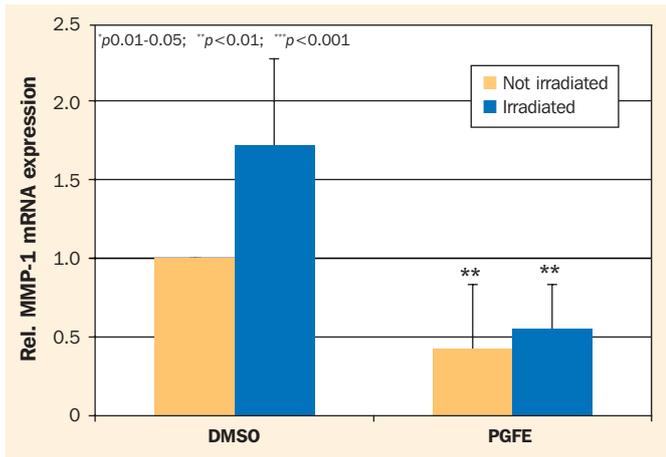


Figure 1: MMP-1 gene expression in primary dermal fibroblasts.

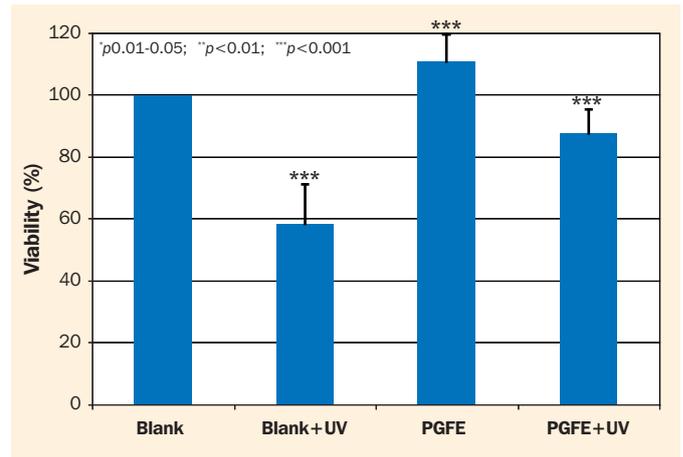


Figure 2: UVA cytotoxicity assay.

In order to obtain a product with precisely these effects, there was a need for the development of a combination of several components into one carrier system. To promote collagen synthesis the stable ascorbic acid derivative sodium ascorbyl phosphate (SAP), already known from literature and used in cosmetics, was chosen.^{9,10} In order to find a new component that reduces MMP-1 synthesis, a screening was carried out on several plant extracts based on a literature research. In this connection, the effects of the chosen extracts on MMP-1 expression in UVA irradiated primary dermal fibroblasts was investigated. At the end of this series of tests, a pomegranate flower extract (PGFE) showed the desired MMP-1 expression-reducing properties.

The aim of this study was to demonstrate that the two active components, sodium ascorbyl phosphate and pomegranate flower extract, could be combined into an effective and safe active ingredient for anti-ageing purposes. Collagen synthesis should be increased by SAP and the breakdown of collagen reduced by the pomegranate flower extract. In this way the balance between collagen synthesis and collagen breakdown should be shifted in favour of collagen synthesis. Wrinkles already present should be reduced and the development of new wrinkles prevented.

Material and methods

● MMP-1 gene expression analysis (real-time RT-PCR)

Selected plant extracts were examined in a real-time reverse transcription polymerase chain reaction (real-time RT-PCR) screening for MMP-1 gene expression-inhibiting properties. Primary dermal fibroblasts were cultivated in 6-well culture plates and treated for 24 hours with 0.1% DMSO (negative control) or extract. In addition, one group was irradiated with 75 mJ/cm² UVA light. Twenty-four hours after irradiation of the cells the mRNA was extracted and the relative MMP-1 gene expression determined using real time RT-PCR.

● MMP-1 synthesis (ELISA)

In order to also confirm the results obtained in the RT-PCR tests at the protein level, collagen production was investigated in normal human dermal fibroblasts (NHDF, Bioalternatives, France). NHDF were cultivated up to senescence (passage 17 (P17)) and then treated with the test substance in 96-well plates for 72 hours. Then the quantity of secreted MMP-1 in supernatant was determined using an enzyme-linked immunosorbent assay (ELISA). A senescent NHDF culture (P17) treated with 10 ng/mL transforming growth factor beta (TGF-β) and an untreated non-senescent NHDF culture (passage 7 (P7))

were used as positive controls. The MMP-1 production of an untreated P17 culture was used as a reference.

● UVA cytotoxicity assay

Normal human dermal fibroblasts (NHDF, PromoCell, Germany) were grown to confluence in 96-well plates and Petri dishes with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) content. Subsequently the DMEM with 10% FCS content was replaced by DMEM with 1.25% FCS content. In addition, 0.1% pomegranate flower extract was added to the medium of selected cultures and then incubated for 24 hours. Half of the cultures were irradiated with UVA. To do this, a UV crosslinker (Vilber Lourmat, Eberhardzell Germany) equipped with UVA lamps (T-8.L UV tube 8W – 365 nm) was used. For irradiation, the culture medium was suctioned off and the cells were covered with PBS. After irradiation with 12.5 mJ/cm² UVA light, the PBS was aspirated off and fresh culture medium was added accordingly with or without the addition of 0.1% pomegranate flower extract. The non-irradiated cells were also treated with PBS and covered with fresh culture medium. Cell irradiation was carried out three times. Between each irradiation there was a 24 hour incubation time in each case. For evaluation, images of the

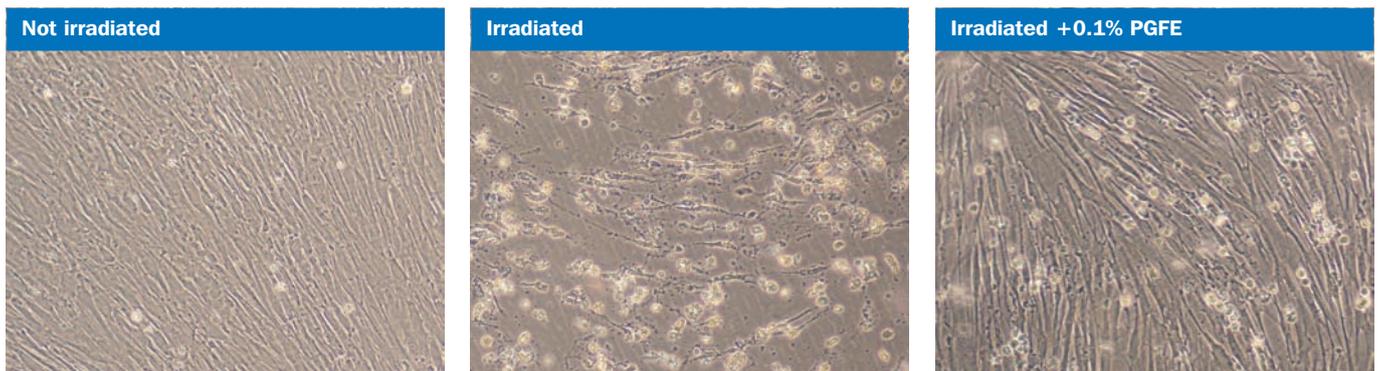


Figure 3: Light microscopic images of NHDF in the UVA cytotoxicity assay.

Petri dish cultures were taken using light microscopy and XTT cytotoxicity tests were performed on the 96-well plate cultures.

● **Product manufacture**

Pomegranate flower extract, sodium ascorbyl phosphate and maltodextrin were mixed and freeze-dried. The portion of pomegranate flower extract was selected based on its activity in the MMP-1 ELISA. In this way the product was standardised with respect to its effectiveness. The freeze-dried end product is called PGFE/SAP in the following.

● **Application formula**

For the *in vivo* tests on product safety and effectiveness, 1% and 5% respectively PGFE/SAP was introduced into a cream formulation (Table 1). The stability of the cream formulation was tested at room temperature and 40°C for 6 months.

● **Tests concerning product safety**

In order to be able to guarantee the safety in use of PGFE/SAP and the cream formulation containing PGFE/SAP, several safety tests were carried out. PGFE/SAP was tested for cytotoxicity (MTT and XTT assays), phototoxicity (according to OECD guideline no. 432) and mutagenicity (Ames test according to OECD guideline no. 471). In addition, an eye irritation test (HET-CAM) was carried out and an allergy certificate in compliance with Directive 2003/15/EC Annex III issued. The cream formulation containing PGFE/SAP was investigated in two different skin irritation tests (SPT and RPT).

● **In vivo studies**

The effectiveness of PGFE/SAP was investigated in two different *in vivo* studies. Study 1 used a test panel of 20 female subjects with Caucasian skin type (phototype I-III). The female subjects, who were aged between 40 and 65 and

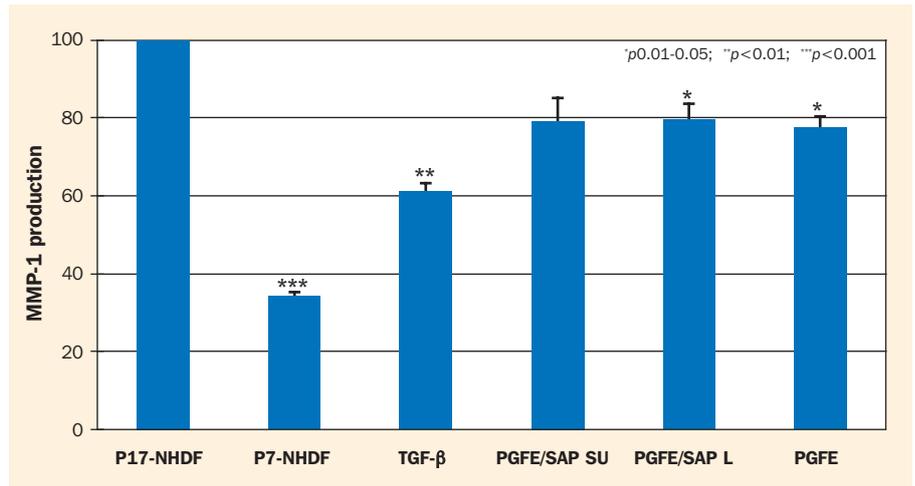


Figure 4: MMP-1 production in senescent dermal fibroblasts.

healthy, took part in the study on a voluntary basis without remuneration. A cream formulation with 1% PGFE/SAP content was applied twice daily for 56 days to the face and the inner side of the forearms. At three points in time (D0, D28, D56) skin thickness was determined by ultrasound and high-resolution facial photographs taken.

In Study 2, a cream formulation with 1% PGFE/SAP content was tested on ten subjects (panel requirements according to Study 1). The cream formulation was applied to the face and tested for 56 days. Similarly to Study 1, facial photos were taken at measurement times D0, D28 and D56. In addition a skin profile was completed from the lateral corner of the eye (crow's feet area).

● **Ultrasonography**

In Study 1, a Derascan C ultrasound machine was used at a frequency of 20 MHz in order to determine the thickness of the upper layers of the skin. The measurements were taken on the inner side of the forearm. For evaluation the number of dark pixels was determined

in the ultrasound images. A reduction in dark pixels was interpreted as an increase in skin thickness.

● **Profilometry (PRIMOS)**

The profilometric measurements in the framework of *in vivo* Study 2 were carried out using the PRIMOS Pico (GF Messtechnik) method in the area around the lateral corner of the eye (crow's feet area). On measurement days D0, D28 and D56, three measurements were taken on each day. To calculate the skin profile and determine the depth of wrinkles, PRIMOS software V5.7, 2010 was used.

● **Visual evaluation (VisioFace Quick)**

In order to guarantee standardised photo documentation, a VisioFace Quick machine was used. The visual evaluation of treatment results was carried out by trained professional staff taking high-resolution images the face (10 MPixel).

Results

Product safety

● **Cytotoxicity**

To evaluate cytotoxicity two series of tests

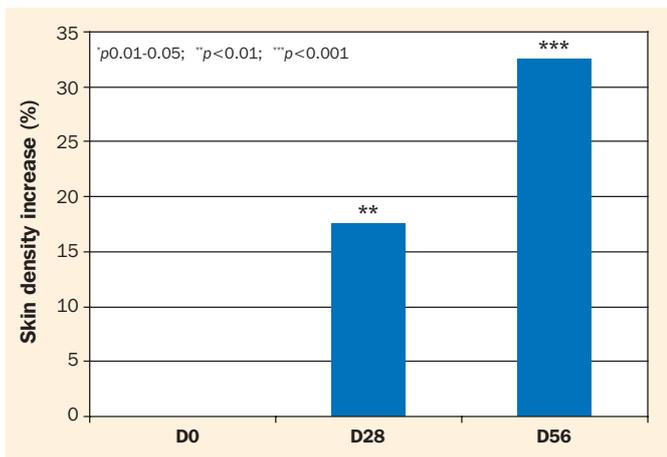


Figure 5: Increase in skin density on the volar forearm.

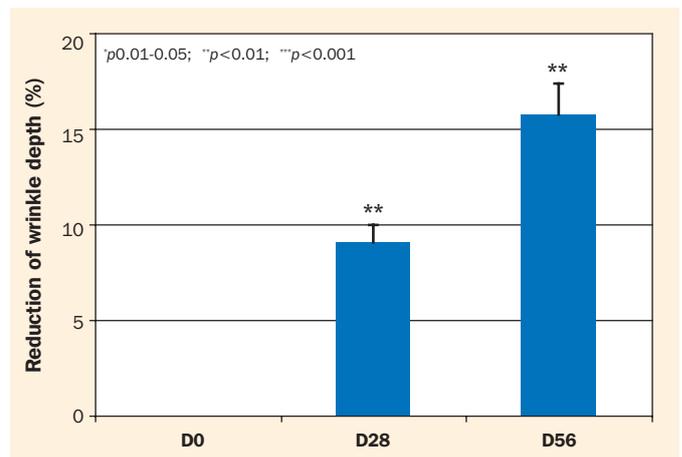


Figure 6: Reduction in wrinkle depth in the lateral corner of the eye (crow's feet area).

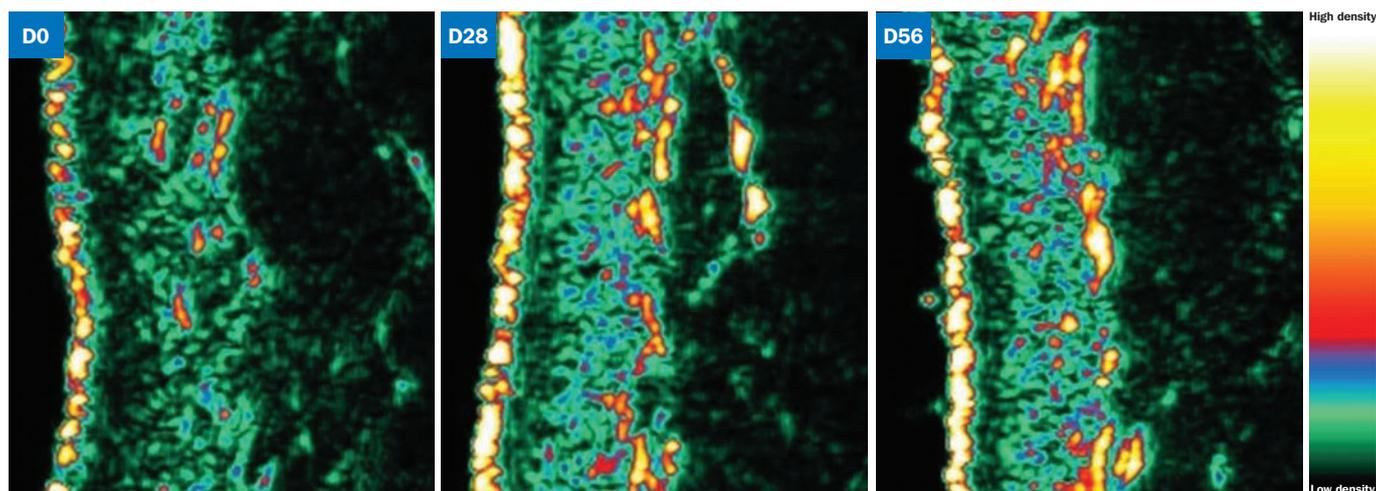


Figure 7: Ultrasonographic images of the volar forearm.

were carried out with different cell types. Up to 2.0% concentration levels PGFE/SAP showed no cytotoxic effect in an MTT assay on normal human dermal fibroblasts and in an XTT assay on NCTC2544 keratinocytes.

● Phototoxicity

In a study on phototoxicity conforming to OECD Guideline no. 432 on mouse embryo fibroblasts (3T3), PGFE/SAP was classified as 'not phototoxic'.

● Mutagenicity (AMES)

The AMES reverse Mutations Assay on *S. typhimurium* and *E. coli* conform to OECD Guideline 471, could not demonstrate any mutagenicity caused by PGFE/SAP.

● Eye irritation (HET CAM)

In order to detect any possible eye-irritating potential of PGFE/SAP, tests were conducted on the chorioallantoic membrane of hens' eggs (HET-CAM). PGFE/SAP was 'practically non-irritating to the eyes' at a concentration level of 1% and at 5% 'slightly irritating to the eyes'.

● Skin irritation (SPT/RPT)

PGFE/SAP was worked into to a cream formulation at levels of 1% and 5% and classified in a single epicutaneous patch test (SPT) as 'non-irritating to the skin'. In a repeated epicutaneous patch test (RPT) the cream formulation containing 1% PGFE/SAP caused no sensitisation of the skin. Likewise, application of a cream formulation containing 5% PGFE/SAP twice a day for 56 days caused no skin irritation or sensitisation.

● Allergy research

Based on an evaluation of allergy-releasing potential according to Directive 2003/15/EC Annex III, PGFE/SAP was classified as non-allergenic.

In vitro

● MMP-1 gene expression analysis (RT-PCR)

The influence of pomegranate flower extract on MMP-1 gene expression was investigated using real time RT-PCR, on irradiated and non-irradiated primary dermal fibroblasts. The results are shown in Figure 1.

Primary dermal fibroblasts were treated with 1 μ L/mL DMSO and 7.11 μ L/mL pomegranate flower extract (PGFE). Twenty-four hours after treatment one group was irradiated with 75 mJ/cm² UVA light. The mRNA was extracted 24 hours after irradiation. All results are shown as a multiple of the non-irradiated DMSO control group. The UVA irradiated cells of the control group treated with DMSO showed a 1.71-fold increase in the expression of MMP-1 mRNA. The MMP-1 mRNA expression in cells treated with PGFE was obviously reduced with and without UVA irradiation. Non-irradiated cells showed 0.42% and UV-irradiated cells showed 0.55% of normal MMP-1 mRNA expression. It should be pointed out that the PGFE treatment reduced not only the MMP-1 gene expression in irradiated cells (45% reduction) but also the MMP-1 gene expression in non-irradiated cells (58% reduction).

● UV cytotoxicity

The UV cytotoxicity test investigated the influence of pomegranate flower extract on the cell vitality of normal human dermal fibroblasts (NHDF) with and without UVA irradiation. The results are shown in Figure 2.

For each series of tests the NHDF of one group was treated with 0.1% PGFE. At the same time a control group was left untreated. Half of the cultures of each group were irradiated with UVA light (12.5 mJ/cm²) three times. There was a 24-hour period between each irradiation.

Then an XTT cytotoxicity test was performed to determine cell vitality 24 hours after the last irradiation. Figure 2 shows the cell viability, calculated from cytotoxicity test results with the untreated non-irradiated control group set as 100%. After UVA irradiation, the untreated cells (Blank+UV) showed a vitality of 59% which corresponded to a vitality deficit of 41%. The cells treated with 0.1% pomegranate flower extract (PGFE+UV) showed clearly higher vitality (88%). The vitality deficit was therefore only 12%. The non-irradiated cells treated with 0.1% pomegranate flower extract (PGFE) showed an increased vitality value of 111%. Figure 3 shows light microscopy images of (a) non-irradiated, (b) irradiated and (c) irradiated cells treated with PGFE 0.1%, 24 hours after the last irradiation with UVA. The non-irradiated NHDF (a) showed a normal appearance. The irradiated cells without extract additive (b) showed severe UV damage (changed cell morphology with many dead cells in the medium). The irradiated culture treated with PGFE (c) demonstrated obviously less UV damage. The majority of cells still showed adhesion and were of a normal appearance. In comparison to culture (b) there were clearly fewer cells to be seen in the medium.

● MMP-1 synthesis (ELISA)

The ELISA was performed in order to demonstrate the MMP-1 inhibiting effect of both the PGFE component and the PGFE/SAP at the protein level and to prove that the PGFE extract as well as two variants of PGFE/SAP produced in different scale productions caused the same MMP-1 inhibiting effect in aged dermal fibroblasts.

Figure 4 shows MMP-1 production quantified by ELISA in senescent dermal fibroblasts. Aged fibroblasts (P17-NHDF) were treated for 72 hours with 10 ng/mL TGF- β , 0.3% PGFE/SAP SU (from Scale-Up methodology 150 kg), 0.3% PGFE/SAP L

(from laboratory methodology 0.5 kg) or 0.1% PGFE. All results shown have been calculated relative to the senescent fibroblast culture control group (P17-NHDF). The MMP-1 production of the non-senescent positive control (P7-NHDF) was 34%, that of the TGF- β treated cells 61% of the control group. All three cell treatments involving PGFE (PGFE/SAP SU, PGFE/SAP L and PGFE) effected production of MMP-1 to be lowered to 80% of the control group's production.

In an identically designed experiment carried out six months previously, laboratory scale-preparations of PGFE extract and PGFE/SAP were tested (results not shown). Both treatments also led to a reduced MMP-1 synthesis of 80%.

In vivo

After successfully passing safety- and stability-tests, a cream formulation containing 1% PGFE/SAP (see Table 1) was used in two *in vivo* studies each with different measurement parameters. In Study 1 skin density was determined on the volar forearms and high-resolution photos of the face were analysed. In Study 2 the depth of wrinkles in the crow's feet area around the lateral corner of the eye was determined and likewise high-resolution facial photos were taken.

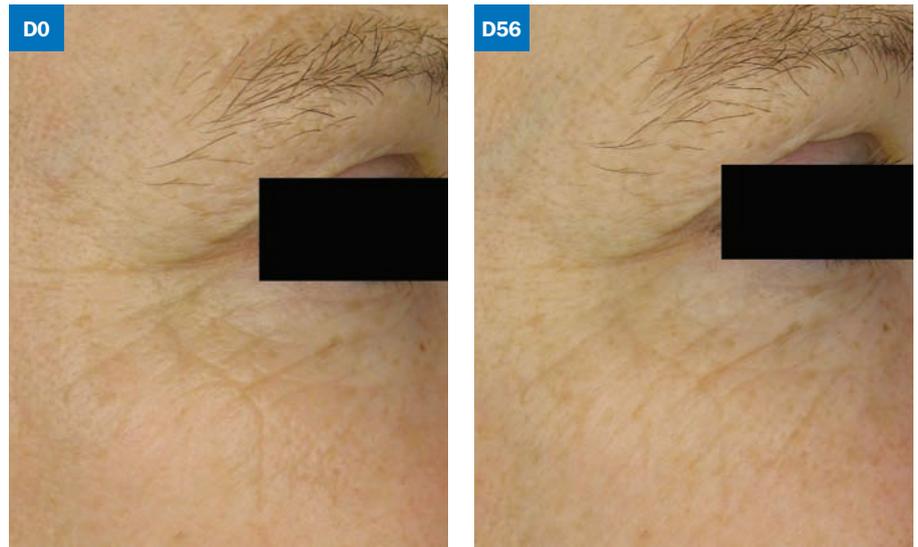


Figure 8: High-resolution photographs of the lateral area around the eye (crow's feet area).

● **Skin density measurement using ultrasonography**

The skin density on the volar forearm was determined using ultrasonography. Measurements were performed before (D0), during (D28) and at the end of treatment (D56) with a cream formulation containing 1% PGFE/SAP. An average increase in skin density of 32.5% over 56 days could be demonstrated. Figure 7 shows the ultrasound images of one of the

test subjects at measurement times D0, D28 and D56. The images were analysed electronically using quantitative determination of dark pixels. The increase in skin density can be seen where there is a decrease in dark pixels. After 28 days, skin density increased by 46.8% and after 56 days by 56.2%.

● **Profilometry (PRIMOS)**

As part of the second *in vivo* study,

profilometric measurements were performed in the area of the outer corner of the eye using the PRIMOS Pico system. Figure 6 shows the reduction of wrinkle depth in the region of the lateral corner of the eye at the starting point (D0), after 28 days (D28) and 56 days (D56) of treatment with a cream formulation containing 1% PGFE/SAP.

After 28 days of treatment a reduction in wrinkle depth could be observed in 90% of the test subjects. The maximum reduction in wrinkle depth measured was 25.5% with an average value of 9.1% after 28 days. After 56 days of treatment, a significant reduction of wrinkle depth was observed in 100% of test subjects. The average reduction in wrinkle depth came to 15.8% and the maximum reduction to 46.9%.

● Visual evaluation (VisioFace Quick)

In both Study 1 and Study 2, high-resolution photos of the face were taken. In order to obtain standardised images in terms of brightness, image size and field of vision, a VisioFace Quick device was used. The pictures in Figure 8 show an example from Study 1 and Figure 9 an example from Study 2.

Figure 8 shows photographs of the crow's feet area at the start (D0) and the end (D56) of Study 1. A clearly visible decrease in the appearance of lines can be observed. Figure 9 shows pictures of the forehead region at measurement times D0, D28 and D56 taken in Study 2. The frown line/forehead wrinkle well recognisable in the centre of the photograph was clearly reduced by treatment with a cream formulation containing 1% PGFE/SAP. In addition an overall smoothing of the forehead area can be seen.

Discussion

The results discussed in this paper demonstrate the safety and effectiveness of the combination of pomegranate flower extract and sodium ascorbyl phosphate. PGFE/SAP exhibited the desired effects in a series of *in vitro* and *in vivo* experiments.

MMP-1 gene expression analysis in primary dermal fibroblasts (Fig. 1) showed an increase in MMP-1 mRNA gene expression evoked by UVA irradiation. This effect is described in literature⁵ and therefore was expected and reproducible. The increased MMP-1 mRNA gene expression in irradiated cells was not only halted but actually reduced by 45% after treatment with pomegranate flower extract. It is worth noting that PGFE also had an effect on endogenous MMP-1 mRNA gene expression, since there was also a 58% reduction in MMP-1 gene expression in PGFE treated non-irradiated cells. Thus



Figure 9: High-resolution photographs of the forehead region.

PGFE appeared to be able to influence not only the extrinsic photoageing process evoked by UVA irradiation but also chronological skin ageing in terms of lowering MMP-1 expression.

In order to demonstrate this effect at protein level, an ELISA quantification of MMP-1 in aged dermal fibroblasts was carried out (Fig. 4). Furthermore it had to be clarified whether pomegranate flower extract activity was still present in the PGFE/SAP after freeze drying. In all the samples containing PGFE MMP-1 synthesis was decreased on average by 20%. This showed that PGFE's properties for inhibiting MMP-1 synthesis had been retained in the freeze drying process and the planned standardisation of PGFE/SAP as regards its activity was successful. The reproducibility of the results after six months showed that PGFE/SAP remained stable and the inhibiting activity in terms of MMP-1 synthesis had not decreased.

The impairment of cell vitality in the UV cytotoxicity test from repeated UVA irradiation was diminished from 41% to 12% by using 0.1% pomegranate flower extract (Fig. 2). This showed that PGFE has cell-protecting properties with respect to UVA-induced impairment of cell vitality.

The anti-ageing effect of PGFE/SAP demonstrated in the *in vitro* tests was confirmed by the *in vivo* studies carried out. The use of a cream formulation containing 1% PGFE/SAP in Study 1 increased collagen thickness significantly (by 32.5%) on the volar forearm (Fig. 5 and Fig. 7) and visibly reduced the appearance of wrinkles around the lateral corner of the eye (Fig. 8). PGFE/SAP also showed an anti-ageing

effect in the second *in vivo* study. The 28-day application of a cream formulation containing 1% PGFE/SAP produced a 9.1% reduction in line depth in the area around the lateral corner of the eye (Fig. 6). After 58 days of application, line depth decreased by 15.8%. An example from this *in vivo* study is given in Figure 9.

The different *in vivo* and *in vitro* tests demonstrated that PGFE/SAP was able to meet the desired requirements in terms of its activity. It was possible to demonstrate MMP-1 inhibition from the PGFE component in the ELISA experiment and the expected increase in collagen synthesis from the SAP component with the help of the ultrasonography results. The results of both effects were visible with profilometric-determined (Fig. 6) and visually-assessed (Fig. 8 and Fig. 9) reduction in line depth. The tests conducted for allergic potential, mutagenicity, phototoxicity as well as skin and eye irritation demonstrated the safety of use of PGFE/SAP. Together with the *in vitro* and *in vivo* data collected on efficacy the result is therefore an anti-ageing product that is very effective and safe to use. PC

- PGFE/SAP is sold under the Cosmtochem trade name Colla-Gain™

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