Combat inflammaging with upcycled mandarin extract

Giovanna Grigolon, Kathrin Nowak, Franziska Wandrey, Fred Zülli – Mibelle Biochemistry

ABSTRACT

'Inflammaging', a term coined by Professor Franceschi two decades ago, refers to a persistent, low-grade inflammatory state that develops with age due to a combination of chronological ageing, intrinsic factors, and external stressors. As we age, the body's capacity to counter inflammation diminishes, leading to this chronic inflammatory state, which contributes to visible signs of ageing skin and age-related pathologies, creating a vicious cycle. In fact, this inflammatory state reduces the energy available to produce essential skin molecules like hyaluronic acid and collagen, levels of which are observed to decrease in ageing skin. Therefore, boosting energy to enhance the production of these vital molecules can significantly benefit the skin.

Both intrinsic and extrinsic stressors contribute to inflammation and accelerate the ageing process by generating reactive oxygen species (ROS).[1] ROS activate the transcription factor NF-κB, which translocates into the nucleus upon activation, and in turn triggers the transcription of various proinflammatory genes, including some encoding cytokines, chemokines, and matrix metalloproteinases (MMPs).[2]

In the skin, this process leads to inflammation and the breakdown of essential extracellular matrix components like collagen, resulting in a loss of skin elasticity and firmness and, ultimately, sagging skin. Consequently, controlling NF-κB activity becomes pivotal in mitigating inflammaging (Figure 1). [3,4]

Upcycled Chios mandarins

The Greek island of Chios hosts a time-honored, organic mandarin cultivation. The gentle climate nurtures an abundance of essential oils and antioxidants in these mandarins, which have yellow-orange skin and soft, slightly orange-coloured flesh.

Despite deviating from today's market standards due to their numerous seeds, Chios mandarins are highly valued for their exceptional and intense aromatic properties.[5] In a traditional, small distillery, both the juice and peel of these mandarins undergo a gentle distillation process to create fragrances.

The remaining thick peel pulp is a unique leftover material, used by Mibelle Biochemistry to extract valuable antioxidants through a gentle, water-based upcycling process, creating a skin-specific active ingredient from these special mandarins (Figure 2).

From literature, it is known that antioxidants derived from citrus fruits have the potential to suppress the immune regulation by NF-κB signaling.[6] We therefore tested the Chios mandarin extract on its potential to combat inflammaging.

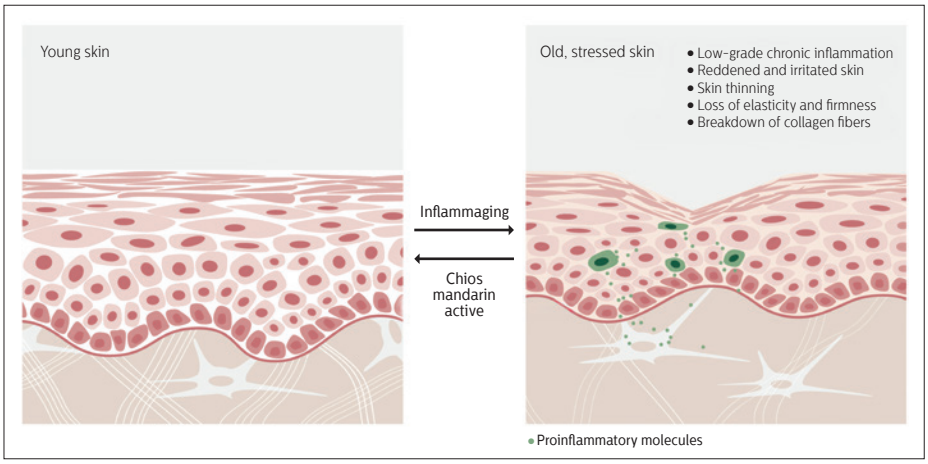


Figure 1: Counteracting inflammaging

Methods

Quantification of NF-κB activation in human dermal fibroblasts

Human dermal fibroblasts were incubated under normal conditions and then acclimated for 24 hours under low-serum conditions. Subsequently, cells were pretreated with either Chios mandarin extract or dexamethasone for 24 hours or left untreated.

Dexamethasone is a reference compound, and it is known for its anti-inflammatory potential. To induce inflammatory stress, PMA (phorbol 12-myristate 13-acetate) and calcium ionophore (CI) were added to the fibroblasts

Figure 2: Chios mandarins

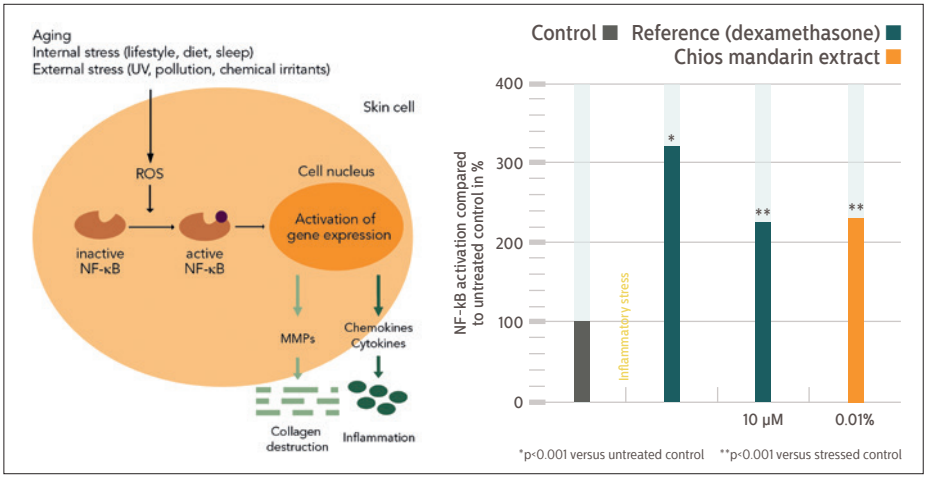


Figure 3: Reduced activation of NF-κB

for an additional six hours. After the treatment, cells were washed and collected. Nuclear proteins were isolated using an extraction protocol. Following this, active NF-κB was quantified.

Gene expression analysis under chronic inflammation in keratinocytes

Human keratinocytes were incubated for 24 hours with Chios mandarin extract or left untreated. Chronic inflammation was then induced in human keratinocytes by the addition of an inflammatory cocktail containing LL-37, calcitriol, and IL-17 for 48 hours. Following this, the expression of candidate genes that are important for the inflammatory response was analyzed.

Fibroblasts treatment with inflammatory keratinocyte secretion

To induce inflammatory keratinocyte secretion, cells were exposed to UV radiation (275 mJ/cm^2 UVB and 2.1 J/cm^2 UVA) and subsequently incubated for 48 hours. Dermal fibroblasts were treated for 24 hours with or without 0.33% and 1% Chios mandarin extract.

After the incubation time, the treatments were refreshed, and the supernatant of the irradiated keratinocytes was added for 72 hours. At the end of the study, the expression of candidate genes that are important for the inflammatory response was analyzed.

Procollagen I secretion

In this assay, skin fibroblasts were treated with or without 0.33% Chios mandarin extract for 24 hours. To induce oxidative stress, 800 µM H_2O_2 was added for two hours.

Following this, the cell culture medium was replaced with fresh medium containing Chios mandarin extract at the concentration used before. After 72 hours of incubation, the secretion of procollagen I was analyzed.

Production of newly formed collagen

Human skin explants were treated topically with a cream containing 0.5% and 1% Chios mandarin extract or a placebo cream. Following seven days of treatment, punches of the skin explants were fixed and prepared for staining.

After Herovici staining, images of the skin explants were captured using brightfield microscopy (Nikon microscope from Nikon Corporation). In the images taken, the collagen fibers were localized in the dermis of the human skin explants. Young collagen is represented in blue, while mature collagen is represented in red magenta.

Clinical studies

We aimed to investigate the impact of our Chios mandarins active *in vivo* on a panel of volunteers with signs of skin redness as well as photo-aging. Twenty women aged between 51 and 68 years (mean age: 60.9 years) were included in this randomized, placebo-controlled clinical study.

The volunteers applied a cream containing 2% Chios mandarins active or a corresponding placebo cream on each side of their face and on two separate areas on the forearms twice daily for 28 days. Facial skin redness was determined at days 0, 7, and 28 using the Spectrophotometer® CM-700d from Konica Minolta, and images were captured using the ColorFace® from Newtone Technologies.

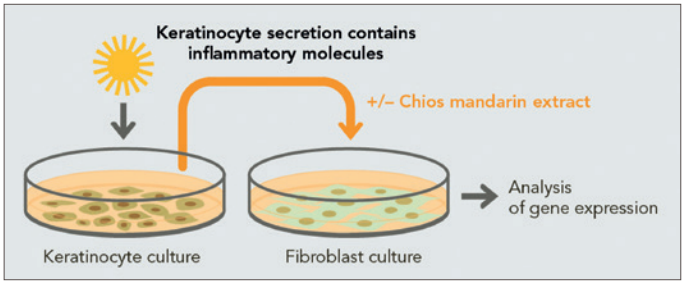
In addition, skin elasticity and skin firmness of the face were analyzed at days 0, 7 and 28 using the Cutometer® dual MPA 580 from Courage and Khazaka, while skin density was measured on the forearms of the volunteers using the Dermalab® ultrasound device from Cortex Technology.

Results and discussion

To investigate the effect of Chios mandarin extract on inflammation, *in vitro* studies were performed using keratinocytes and dermal fibroblasts.

Pretreatment of human dermal fibroblasts with the Chios mandarin extract significantly reduced the activation of NF-κB to the same extent as the anti-inflammatory reference compound dexamethasone, showing that our Chios mandarins active suppresses the NF-κB signaling pathway (Figure 3).

For the keratinocyte assay, cells were treated with an inflammatory cocktail to stimulate chronic inflammation in the presence and absence of Chios mandarin extract. Analysis of genes important for the inflammatory response revealed that co-treatment with Chios mandarin extract decreased the expression of inflammatory markers in keratinocytes.



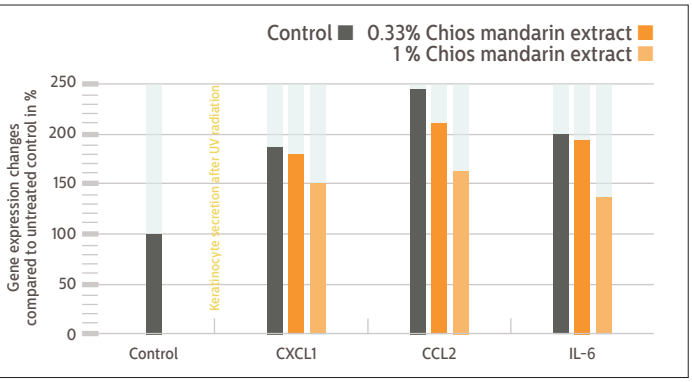


Figure 4: Reduced inflammatory response

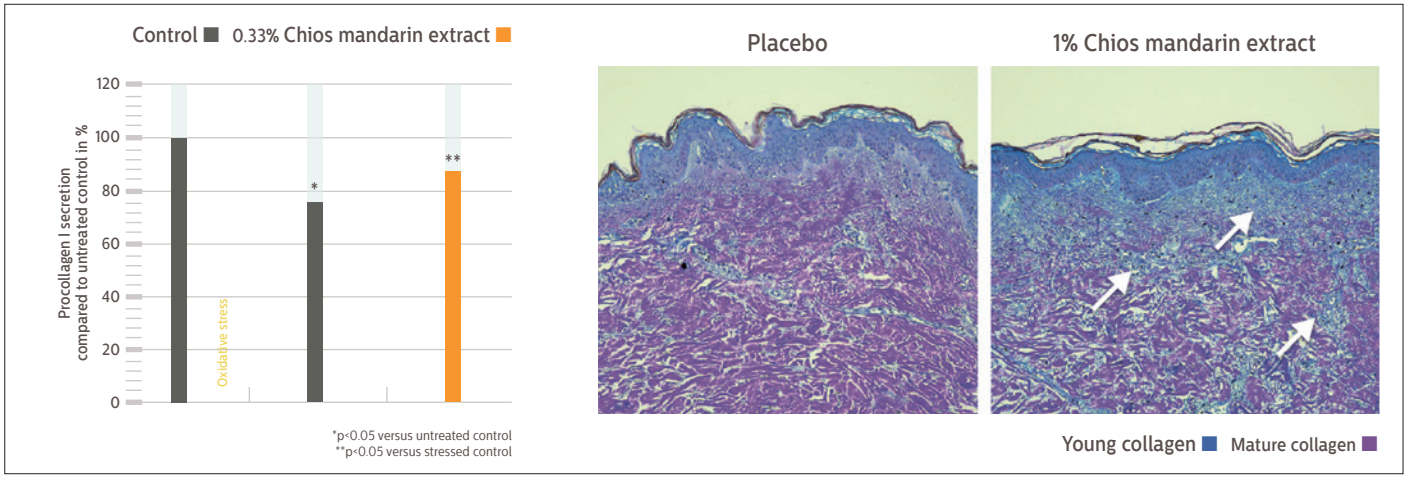


Figure 5: Improved Procollagen I secretion and production of newly formed collagen

In a second assay, dermal fibroblasts were treated with the secretion of UV-stressed keratinocytes to simulate inflammaging conditions, in the presence and absence of the Chios mandarin extract. Again, analysis of genes showed that the inflammatory response was reduced by treatment with Chios mandarin extract representing an anti-inflammatory effect (Figure 4).

Since the inflammatory response was reduced in keratinocytes and dermal fibroblasts, we wanted to test whether Chios mandarin extract influenced collagen expression and deposition. Therefore, dermal fibroblasts were stressed with H_2O_2 in the presence or absence of Chios mandarin extract.

Oxidative stress decreased the secretion of procollagen I whereas treatment with Chios mandarin extract counteracted this effect and increased procollagen I release compared with the stressed control.

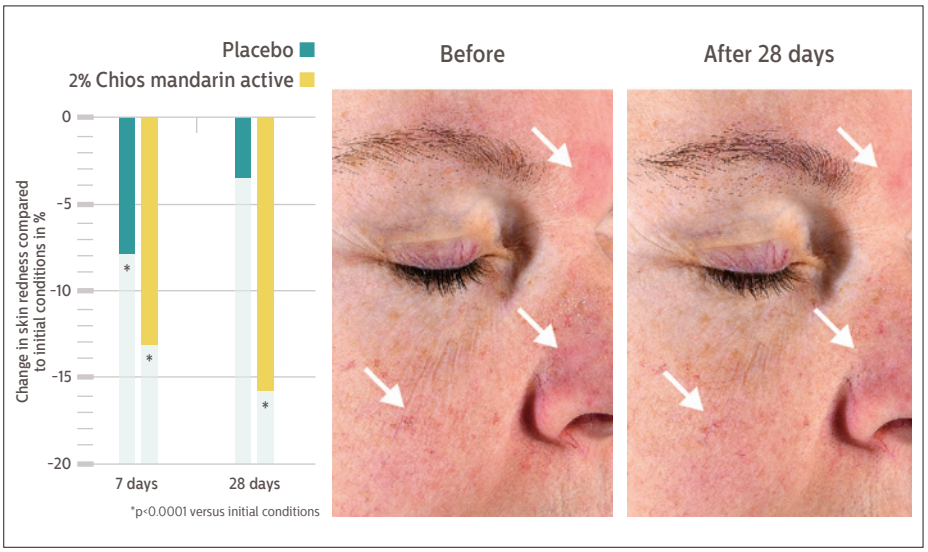


Figure 6: Reduction of skin redness

In addition, an *ex vivo* study was performed on skin explants with a cream containing 0.5% or 1% Chios mandarins active or the corresponding control. The treatment with the Chios mandarins active for seven days resulted in a visible increase in the ratio of young to mature collagen (Figure 5).

To elucidate the calming and rejuvenating effect of our Chios mandarins active, a randomized placebo-controlled clinical study was conducted on a panel of volunteers with signs of skin redness as well as photo-ageing.

Twenty women aged between 51 and 68 years (mean 60.9 years) applied a cream containing 2% Chios mandarins active (INCI: Citrus Reticulata Extract/Citrus Reticulata (Tangerine) Extract (and) Glycerin (and) Pentylene Glycol (and) Aqua/Water, CALMandrin™) or a corresponding placebo on each side of their face and on two separate areas on the forearms twice daily for 28 days.

Treatment with the Chios mandarins active led to a visible improvement in skin redness by -15.9% compared to initial conditions. Interestingly, facial skin redness was already decreased after seven days of Chios mandarins active treatment demonstrating a fast-acting effect on reddened skin (Figure 6).

In addition to skin redness, the effect of our Chios mandarins active on signs of ageing was investigated on the same panel of volunteers. Treatment with 2% Chios mandarins active increased skin elasticity by 29.5% and 29.3% after seven and 28 days compared to initial conditions.

Furthermore, the Chios mandarins active enhanced skin firmness by 13.9% and 16.9% after 7 and 28 days, respectively. Only seven days of Chios mandarins active application strongly improved skin elasticity and skin firmness, highlighting a rapid improvement of the skin parameters.

Besides skin firmness and elasticity, 2% Chios mandarins active significantly improved skin density measured on the forearm by 11.1% after 28 days of treatment compared to initial conditions (Figures 7 and 8).

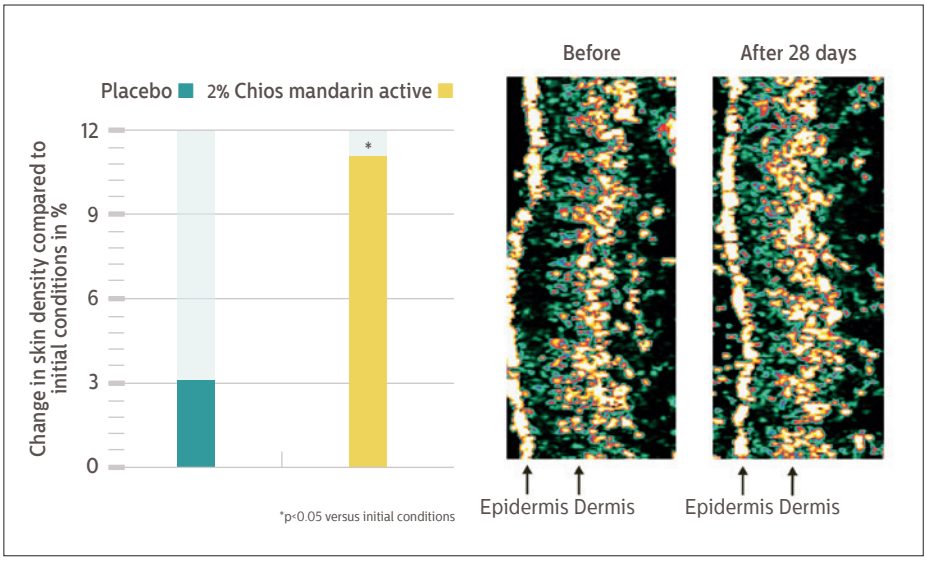


Figure 7: Improvement of skin density

Conclusion

Thanks to its anti-inflammaging effect, the here described active ingredient derived from Chios mandarins markedly increases the firmness and density of the skin. This upcycled elixir is based on the peel paste of organic mandarins discarded from the distillation process of fragrance production. The mandarins are grown exclusively in historic orchards on the Greek island of Chios.

To summarize, our Chios mandarins active counteracts the inflammatory immune response that results from both chronic ageing and extrinsic stress to win the race against the inflammaging-induced signs of skin ageing. **PC**

References

1. Pilkington SM, Bulfone-Paus S, Griffiths CEM, Watson REB. Inflammaging and the Skin. *J. Invest. Dermatol.* 2021;141(4S):1087-95
2. Pillai S, Oresajo C, Hayward J. Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation - a review. *Int. J. Cosmet. Sci.* 2005;27(1):17-34
3. Haque A, Woolery-Lloyd H. Inflammaging in Dermatology: A New Frontier for Research. *J. Drugs Dermatol.* 2021;20(2):144-9
4. Xia S, Zhang X, Zheng S, Khanabdali R, Kalionis B, Wu J et al. An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment. *J. Immunol. Res.* 2016;2016:8426874
5. Varzakas T, Stampelos X, Manolopoulou E. Changes in physico-chemical characteristics of mandarin (common Mediterranean cultivar) Chiotiko (Citrus deliciosa Tenore) from Chios Island in Greece - evaluation of antioxidant capacity and flavonoid content of citrus peels. *International Journal of Postharvest Technology and Innovation.* 2013;3(2)
6. Ren H, Hao J, Liu T, Zhang D, Lv H, Song E et al. Hesperetin Suppresses Inflammatory Responses in Lipopolysaccharide-Induced RAW 264.7 Cells via the Inhibition of NF-kappaB and Activation of Nrf2/HO-1 Pathways. *Inflammation.* 2016;39(3):964-73

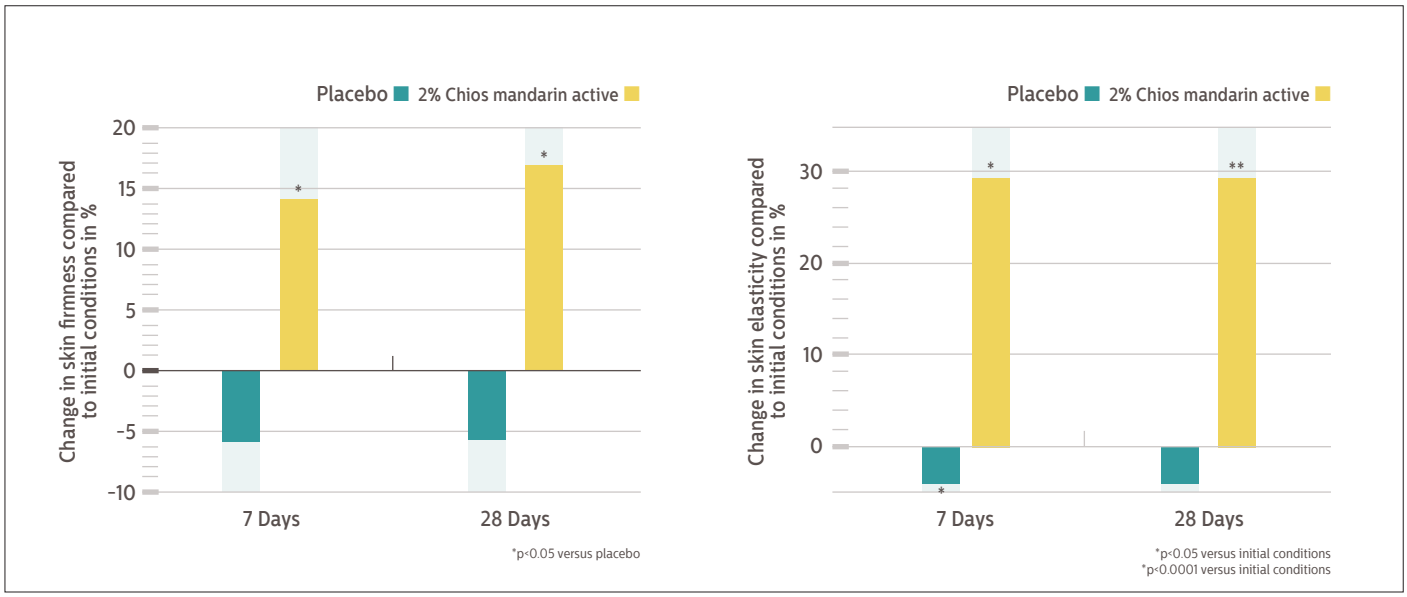


Figure 8: Improvement of skin firmness and elasticity