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Natural alternatives for retinoic-acid-like anti-ageing

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Natural alternatives for retinoic-acid-like anti-ageing

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Abstract

Use of vitamin A derivatives - the retinoids - is common in cosmetic formulations targeting the anti-ageing area. While retinoid acid is banned in the European Union as a cosmetic ingredient, other retinoids such as retinol are permitted. However, retinoids can provoke unwanted effects such as dry and itchy skin, especially when exposed to sunlight. Here we show that an alternative, namely natural bioflavonoids from *Maclura cochinchinensis*, is able to bind the target receptors of retinoic acid and create retinoid-acid-like efficacy *in vivo*.

Use of retinoic acid is the most powerful anti-acne treatment we know so far. It re-programmes sebocytes and keratinocytes to regulate their genes back to the normal level.[1] Thus, sebum production is reduced and hyper-keratinisation is diminished. However, retinoic acid is banned for cosmetic use in the EU and therefore, other vitamin A derivatives are used (retinoids). These derivatives are themselves inactive but are converted to retinoic acid in the tissues.[2] Besides the use in anti-acne products, retinoids are powerful anti-ageing active ingredients as it was found that they have greater efficacy in wrinkle reduction and skin smoothing.[3] Obviously, the use of retinoids has an influence on the biochemistry and metabolism of skin cells. They regulate numerous genes important for the skin's appearance. For example, collagen genes are upregulated and genes responsible for fatty acid synthesis and inflammation are downregulated. In this way, retinoids not only reduce skin impurities but also reduce wrinkles and fine lines leading to a rejuvenation effect.

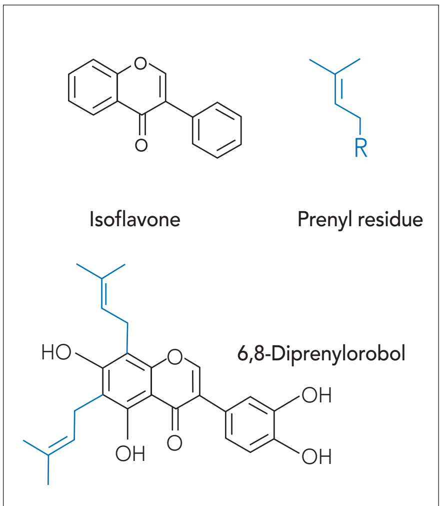

Figure 1: *Maclura cochinchinensis* leaves contain several bioflavones in form of prenylated isoflavones. Right: 6,8-Diprenylorobol consists of the isoflavone core building block (black) with two prenyl side chains (blue) and four hydroxyl groups (grey) attached to the aromatic rings.

However, retinoids may lead to dry skin due to the sebum regulating function, which may also lead to skin irritations in some circumstances. Therefore, the cosmetic industry is seeking new mild ingredients with retinoid function and excellent skin compatibility. As an alternative, bioflavonoids from *Maclura cochinchinensis*, the active principle of Seboclear-MP, can fill this gap. Originally designed to treat acne-prone skin, the bioflavonoids of Seboclear-MP (INCI: Propanediol, Bioflavonoids) are able to bind to the nuclear receptors RAR (retinoic acid receptor) and RXR (retinoic X receptor) to turn back the cellular ageing clock.[4]

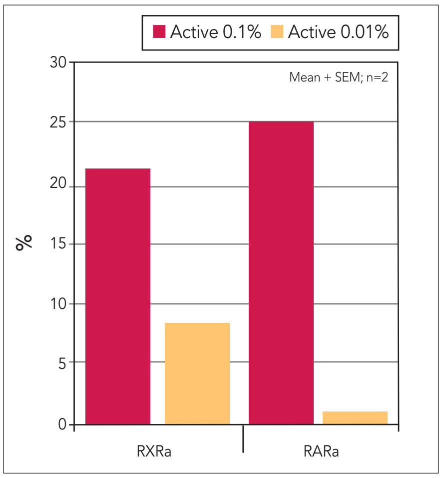

Figure 2: Bioflavonoids from *Maclura cochinchinensis* possess retinol-like activity. A concentration-dependent binding to the receptors sensitive to retinoid acid (RAR and RXR) was observed. The assay suggests an activation of the gene expression of RAR/RXR-controlled genes upon application of the active ingredient.

The bioflavonoids from *M. cochinchinensis* are isolated by extraction of the leaves of the shrub with propanediol and further purification to produce Seboclear-MP. *M. cochinchinensis*, also known as cockspur thorn, grows commonly in the forests of Southeast Asia but is also widespread in Northern Australia and East Africa. It is a branched, thorny shrub, rambling or even climbing on neighbouring vegetation. In the days before synthetic dyes, the plant was used in the traditional 'soga-batik' in Indonesia. The fruits of *M. cochinchinensis* are edible and the young leaves of the plant can be eaten raw. The extract from the roots of *M. cochinchinensis* is known as Chuanposhi in China; it is used in folk medicine for the treatment of humid jaundice, gastric carcinoma, dysmenorrhea, scabies and bruising.[5] Extensive investigations have attempted to characterise the secondary plant metabolites in the extracts of the roots of Maclura. However, little was known about the active ingredients in the leaves of the shrub. Seboclear-MP is the extract of *M. cochinchinensis* leaves with propanediol. We could identify the prenylated isoflavones isolupalbigenin, 6,8-diprenylorobol and 6,8-diprenylgenistein as main ingredients. They have in common the isoflavone core, with two prenyl residues attached at different positions on that core (Fig 1). Isoflavones, a subclass of the bioflavonoids, are secondary plant metabolites that defend the plant against pathogens. They are typically produced by the *Fabaceae* plant family (clover, beans). Highly processed foods made from

Untreated control | Propanediol 0.1% | SEBOCLEAR™ MP 0.1% | Fibroblast control

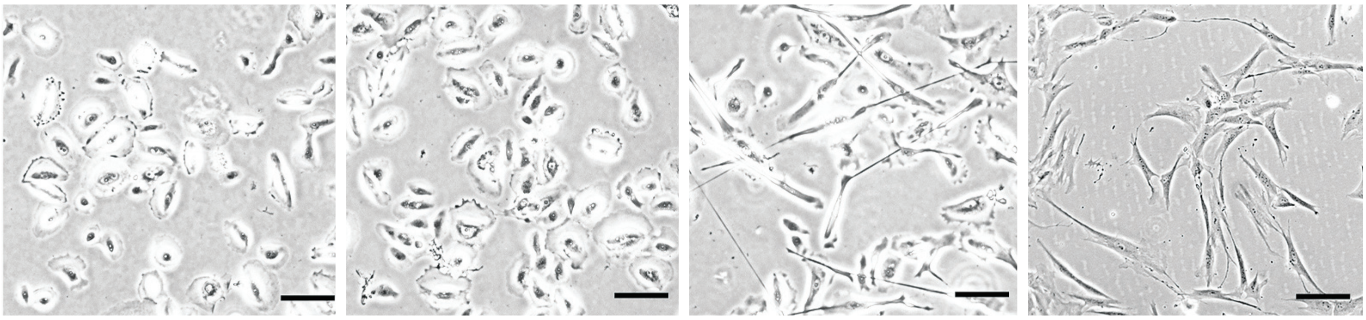

Figure 3: Bioflavonoids from *Maclura cochinchinensis* change the cell morphology of sebocytes. Untreated cells show the typical roundish form of lipid-producing sebocytes. The addition of propanediol has no effect. Treatment with 0.1% active ingredient transforms the cell morphology into a fibroblast-like appearance typical for motile precursor cells.

legumes like soya beans have elevated levels of isoflavones (tofu or fermented miso, for example). Although *Maclura* is a member of the *Moraceae* family of plants (mulberry), the leaves contain high amounts of isoflavones. Bioflavonoids are known to bind nuclear receptors regulating gene expression. Here we investigated their function as retinoic acid like effector molecules in ageing skin.

Methods

Binding of bioflavonoids to the RAR and RXR receptors

To investigate the potential of *Maclura cochinchinensis* bioflavonoids for activating the retinoic acid receptor / retinoid X receptor complex (RAR/RXR), a ligand replacement assay (agonist) with radioactively labelled [^{3}H] 9-cis retinoic acid bound to human recombinant RAR and RXR receptors was performed. For RAR, 1 nM ligand was used, for RXR, 5 nM were used. Put more simply, the addition of agonist (0.01 % or 0.1 % bioflavonoids) will displace the radioactively labelled [^{3}H] 9-cis retinoic acid from the ligand binding site of the receptors. Liberated [^{3}H] 9-cis retinoic acid is detected by scintillation counting[6] after 180 minutes for RAR and after 60 min for RXR.

Re-programming sebocytes

To visualise the effects of *Maclura cochinchinensis* bioflavonoids on cultured primary sebocytes a bright-field microscopic study of the cell morphology was performed. Primary sebocytes from facial location were cultivated in differentiation medium. Terminal differentiation was checked by labelling with keratin-7 antibody and staining with a CY3 labelled secondary antibody. The differentiation was confirmed by FACS analysis. For the viability assay, cultured primary sebocytes were incubated with the tetrazolium salt WST-1, which is converted by the mitochondrial succinate-tetrazolium reductase system into formazan. Formazan can be detected at 450 nm in a spectrophotometer. The colour conversion can only take place in living and metabolically active cells. The assay is comparable to the MTT assay.

Rejuvenation effect on ageing skin

The *in vivo* study has been performed in accordance with the principles of good laboratory practice (GLP), good clinical practice (GCP), and in compliance with the quality assurance system requirements. The study was conducted in accordance with the World Medical Association's Declaration of Helsinki. All study participants signed a written informed consent at the beginning of the study.

A double-blind, placebo controlled, randomised study on 21 female subjects, aged 45 – 64 years (average 56.9) and with healthy, Caucasian skin was performed. The study subjects with signs of ageing skin applied the placebo or verum formulation with 3% bioflavonoids from *Maclura cochinchinensis* in a hemi-face approach twice daily. Each study participant was her own control. The study duration was 3 months with measurements at day 0, day 28 and day 84. Firmness and elasticity of the periocular region were assessed with a standard cutometer (MPA 580, dual, 2 mm probe). Wrinkles in the nasolabial and crow's feet area were determined using a PRIMOS 3D imaging system. The visible appearance of wrinkles and skin texture were assessed by objective evaluation by a dermatologist. The wrinkle appearance was assessed by using the crow's feet wrinkle grading score (WGS), a photo grading scale of 8 degrees,[7] whereas the wrinkle appearance in the nasolabial area was assessed by using the nasolabial wrinkle grading system (scale of 5 degrees).[8] The skin texture parameters of evenness, roughness and

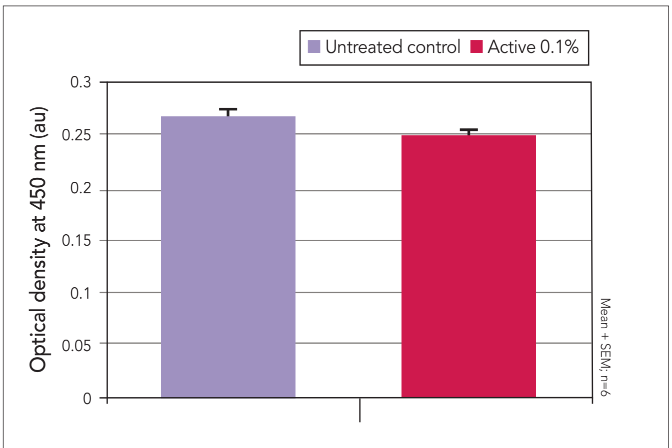

Figure 4: Treatment with bioflavonoids from *Maclura cochinchinensis* has no adverse effect on the viability of sebocytes. This data confirms the good compatibility of the active ingredient and supports the hypothesis of a dedifferentiation of sebocytes as explained in Figure 3.

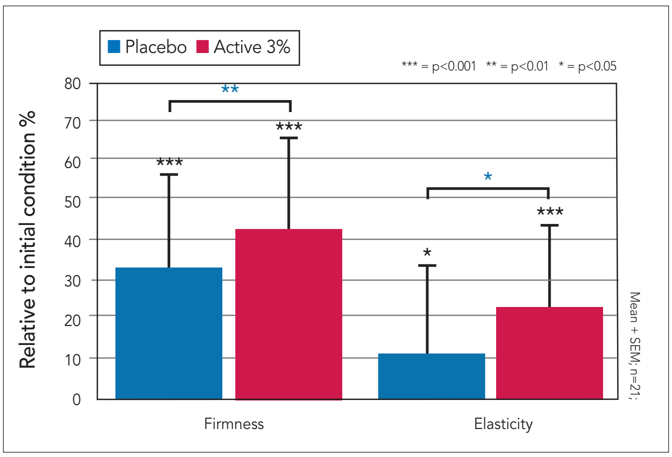

Figure 5: Bioflavonoids from *Maclura cochinchinensis* rejuvenate the skin. Firmness and elasticity are significantly improved above the placebo by up to 43 % over initial condition. Wilcoxon signed-rank test. Statistical values in black refer to comparison with baseline while values in blue refer to placebo.

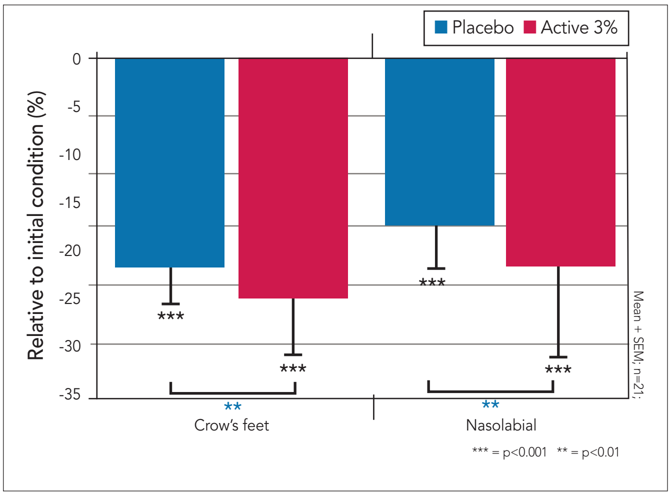

Figure 6: Bioflavonoids from *Maclura cochinchinensis* significantly reduces the number of wrinkles. After 84 days of application, the wrinkle counts in the crow's feet and nasolabial areas decreased significantly above the placebo by up to 42 %.

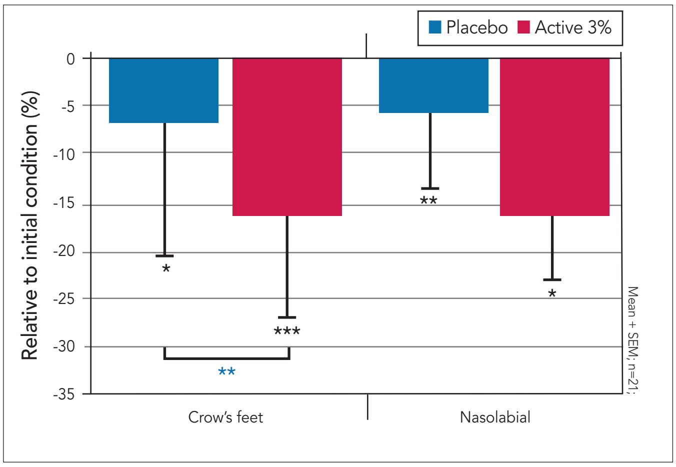

Figure 7: Bioflavonoids from *Maclura cochinchinensis* significantly reduce wrinkle volume. After 84 days of application, the wrinkle volumes in the crow's feet area decreased significantly by 16 %. Wilcoxon signed-rank test. Statistical values in black refer to comparison with baseline, while values in blue refer to comparison with the placebo.

suppleness were assessed using a 9-degree scale ranging from very fine skin texture to very coarse skin texture.

Results

Bioflavonoids from *Maclura cochinchinensis* have retinoic acid-like efficacy *in vitro*

To achieve a retinoic acid-like efficacy, it is obvious that the key nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR) have to be activated to regulate their corresponding target genes. In our experiments the *Maclura cochinchinensis* bioflavonoids replaced bound radio ligand from the nuclear retinoic acid receptors RAR and RXR in a concentration-dependent manner. The active ingredient at 0.1 % is capable of binding the RAR by 25% and the RXR by 21%. Although this value is just touching the border of significance in this assay, a positive gene regulation of retinoic acid activated genes and as such, a retinoic-acid-like activity on the part of the active ingredient can be deduced from these results.

The morphology of sebocytes treated with the active ingredient differed significantly from untreated or propanediol-treated cells (Fig 3) that show the typical roundish cell morphology of terminally differentiated sebocytes. Instead, we observed a flattened, fibroblast-like cell shape for the cells treated with the bioflavonoids (for comparison see Fibroblast Control in Fig 3). Sebocytes are generated in the germination layer of the sebaceous duct from non-differentiated precursor cells with a flattened cell shape. During maturation, they start a slow apoptotic process to become sebum-producing cells and finally burst to release the sebum into the sebaceous duct. Remarkably, the viability of sebocytes treated with 0.1% active ingredient is not distinguishable from untreated control cells (Fig 4). As such, there is no indication of any cytotoxicity. A possible explanation of the phenotype is a dedifferentiation process as described for sebocytes treated with epidermal growth factor (EGF). EGF shuts down intracellular lipid synthesis almost completely and acts as a suppressor of sebum production.[9] Sebocytes treated with EGF have comparable cell morphology as can be seen by treatment with the active ingredient. We assume that the sebocytes are reverted into an earlier differentiation stage, similar to the sebocyte precursor cells that do not produce sebum. This finding suggests that the bioflavonoids act very similarly compared to retinoids, of which the main activity is also to shut down fatty acid synthesis in sebocytes.

Bioflavonoids from Maclura *cochinchinensis* are effective skin-rejuvenating actives

The results of the *in vivo* study confirmed a retinoic-acid-like mechanism of action to support skin rejuvenation. In a double-blind, hemiface, placebo-controlled study with 21 female study participants, the skin firmness and elasticity were significantly improved after 84 days of application of 3% active ingredient (Fig 5). The firmness increased significantly by 43% compared to the initial condition, being also significant over the placebo. A similar result was obtained for elasticity. Elasticity was improved by 23%, also significant over baseline and the placebo. The efficacy of the active ingredient outperformed the placebo by 100%. The result indicates a positive impact on the dermal gene expression for collagens and elastin, the most important structural proteins increasing the skin's firmness and flexibility.

An increased amount of collagen and elastin should translate into reduced signs of ageing. Indeed, the wrinkle count as analysed by PRIMOS decreased by 42% in the crow's feet area and by 36 % in the nasolabial area at 3% active ingredient. Both improvements were significant over the placebo and the initial condition. In parallel, the wrinkle volumes in both areas decreased by 9 – 16% (Fig 7). Roughness in the periorbital region decreased significantly over the placebo by 6.7% (not shown).

An impressive instrumentally achieved improvement in skin firmness, elasticity and wrinkle reduction parameters does not automatically result in a visible reduction of the signs of skin ageing, which is the desired task for a cosmetic formulation claiming anti-ageing efficacy. For this, the instrumentally evaluated parameters were confirmed by visual dermatological inspection of the wrinkles with corresponding wrinkle grading scales. The general skin appearance was assessed using an anti-ageing efficacy grading system evaluating skin evenness, roughness and suppleness. After 84 days, 3% of the active ingredient visibly decreased the wrinkle grading score by 20% in the crow's feet area and 24.6 % of the nasolabial fold. As can be seen in Figure 8 and 9 the reduction of the wrinkles in the crow's feet area and the improvement of the nasolabial fold are the dominant parameters where the active ingredient outperforms the placebo formulation over time. The improvement was significant over both the initial condition and the placebo. This is in accordance with the activity profile of a classic retinoid treatment.

In addition, application of 3% active ingredient led to a significant improvement in the anti-ageing parameters of evenness, roughness and suppleness over initial condition after 28 and 84 days. Evenness reached significance over the placebo after 84 days (Fig 9).

Conclusion

Cosmetic treatments with retinoic acid are prohibited in the European Union and may only be prescribed as treatments by dermatologists. Because of this, the cosmetic industry uses other vitamin A derivatives such as retinol, which, according to recent study data, can have similar benefits but is less active than retinoic acid.[10] However, retinoid treatment can have side effects such as dry skin accompanied with skin reddening and even itchy skin. The latter may be due to the TRPV1 receptor, which is known to be activated by retinoids and which causes prolonged inflammatory states of the skin as well as sensory hypersensitivity.[11] Therefore, mild treatments taking advantage of the mode of action without the unwanted irritant potential of retinoic acid are highly appreciated. Vitamin A and related retinoic acid derivatives activate the RAR/RXR receptor complex. This regulates a number of genes that control sebum production of the sebaceous gland and the proliferation of keratinocytes. Regulation of the RAR/RXR system is a long-term approach, which is considered to be most effective in the reduction of acne but also in anti-ageing. Active ingredients, which can activate the RAR/RXR receptor complex to facilitate the correct gene expression of the cells without creating unwanted effects by activating several skin receptors, are highly appreciated.

Seboclear-MP, consisting of bioflavonoids from *Maclura cochinchinensis* is a new natural cosmetic active ingredient filling this gap: Besides being an excellent anti-acne active ingredient (not shown here), it brings the same anti-ageing efficacy as a retinoid without any sign of unwanted effects. *In vitro* toxicological investigations did not reveal any deleterious effects. The formulation with 3% Seboclear-MP was very well tolerated in multiple *in vivo* studies with 20 – 50 subjects, each aged 16 – 65 years. The *in vitro* data presented here provide strong evidence that the bioflavonoids from *Maclura cochinchinensis* are able to generate the same mode of action as is described for retinoic acid treatment without interfering with the vitality of the cells. Multiple bioflavonoids can bind to oestrogen receptors and this finding raises the suspicion of potential endocrine disruptive function. It is worth mentioning that no significant binding to the oestrogen receptors was found for the bioflavonoids of *Maclura cochinchinensis*, indicating that endocrine disruption cannot take place. Development of new cosmetic active ingredients never stops and with Seboclear-MP we are entering a new retinoid-free anti-ageing approach to make possible unstrained, natural skin rejuvenation. PC

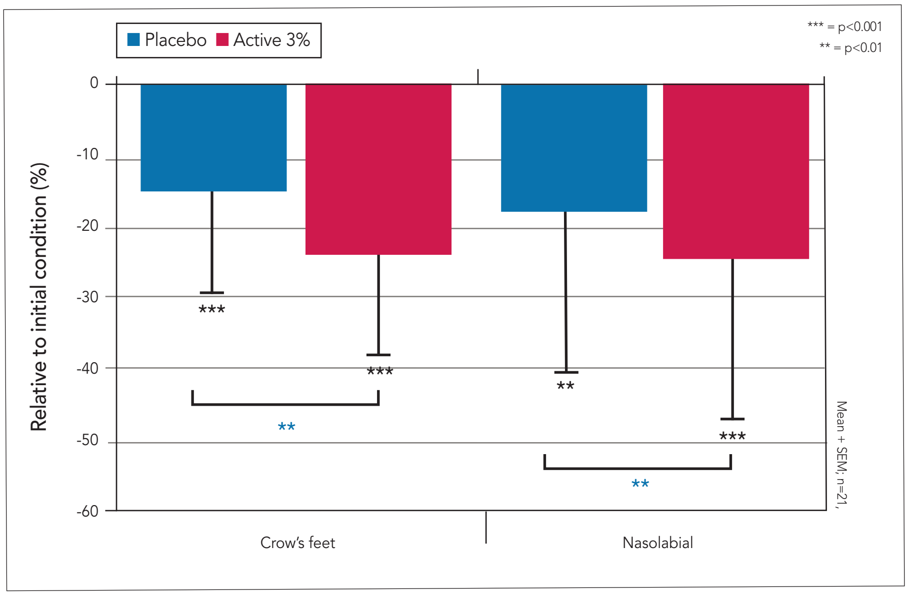

Figure 8: Bioflavonoids from *Maclura cochinchinensis* visually improved the wrinkle appearance as judged by a dermatologist. Improvements in the crow's feet area and of the nasolabial fold were highly significant over baseline and the placebo. Wilcoxon signed-rank test. Statistical values in black refer to comparison with baseline while values in blue refer to comparison with the placebo.

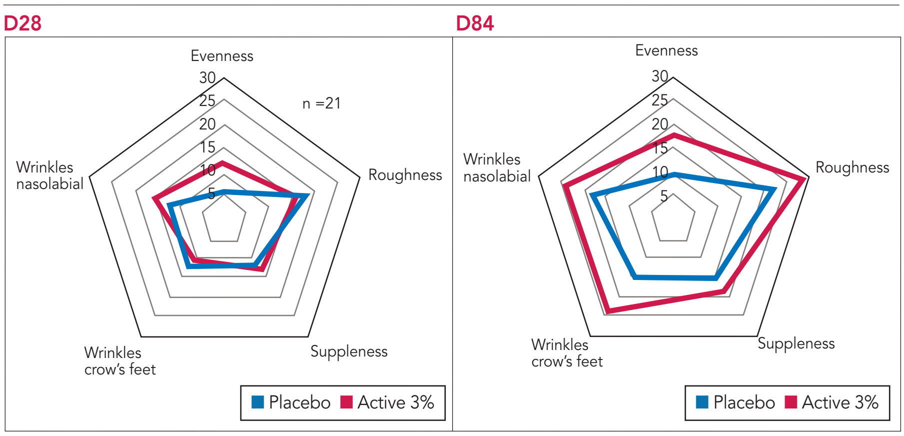

Figure 9: Development of anti-ageing parameters over time. Bioflavonoids from *Maclura cochinchinensis* outperform the placebo in all visually evaluated parameters like skin evenness, roughness, suppleness and wrinkle appearance in the crow's feet area and the nasolabial fold (detailed in Figure 8 for these two parameters). All improvements for the active were statistically significant for baseline and evenness. The wrinkles parameters were also significant for the placebo (D84).

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