A multi-sensorial active ingredient to reduce stress and improve skin condition

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Abstract

Background: The recently discovered olfactory receptors on skin cells paved the way to develop cosmetic active ingredients which stimulate those receptors and drive keratinocyte differentiation and maturation. As such, scents can not only stimulate the brain and act de-stressing but can also act positively on the skin.

Methods: A scenting cosmetic active ingredient from the resurrection bush *Myrothamnus flabellifolia* (INCI: Caprylic/Capric Triglyceride, Myrothamnus Flabellifolia Leaf/Stem Extract) was used for studies stimulating the mood of test subjects and to investigate the effects on skin. Characterisation of the scent profile has been done with a panel of 12 trained people. The composition of the essential oil fraction was determined by GC-MSD. Double-blind, placebo controlled studies were performed (n = 25 - 75). Mood parameters were evaluated by hormone measurements of saliva, questionnaire and EEG recording. Skin parameters were evaluated with standard equipment. Corneometer values were mapped on the images of representative faces by means of computer aided colour mapping. Investigation of bitter taste receptor activation on keratinocytes was assessed by measuring the calcium influx and the cAMP level.

Conclusion: Myrothamnus extract was able to improve the mood of study participants using an emulsion containing 3 % of the active. It further improved skin parameters like hydration, TEWL and anti-ageing parameters. The mode of action combines subconscious smelling via olfactory sensory neurons in the nose and activation of bitter receptors on keratinocytes. This combination is the first-of-its-kind approach of a cosmetic active ingredient to destress the mind and skin at the same time.

Keywords: Stress, olfactory receptor, bitter taste receptor, cortisol, hydration mapping

Introduction

Stress triggers very old hormonal programs in the body that are intended to ensure the organism's survival. In acute stress, the so-called "fight or flight" reaction occurs. The stimulus activates the limbic system. Via the amygdala, the center of emotional reactions and memory formation, the pituitary gland is activated. This results in the release of adrenaline and cortisol, both stress hormones that flood the entire body. There is increased alertness, increased heart rate and release of carbohydrates from the liver. All mechanisms that prepare for rapid flight or attack. The body is ready to deliver peak performance. These processes are controlled by the sympathetic nervous system. In contrast, the parasympathetic nervous system controls calming reactions in the body [1]. Stimulation of the parasympathetic nervous system calms the heartbeat, stimulates rest and relaxation and ensures general relaxation. In normal everyday life, both systems are activated alternately as an expression of the circadian rhythm [2]. While the autonomous nervous system is completely driven by the circadian rhythm, the central nervous system is only indirectly influenced. Chronic stress can upset this sequence. The continuous increase of adrenaline and cortisol keeps us in a constant state of alert. Although cortisol is an anti-inflammatory hormone, a permanent increase leads to the opposite effect in the skin: collagen is degraded, chronic inflammatory conditions occur and the skin barrier is weakened [3, 4]. To keep the mind and skin healthy, good stress management is beneficial.

Positive emotions are an important element in stress management. These are in a complex field of action between conscious decisions, social relationships and experiences. Our emotional image of the world develops throughout our lifespan and is modulated by our environment and social interaction. Over time we learn how activities, strategies and interventions can help us to have positive emotions. They are an integral part of being happy and making us feel well [5]. Since positive emotions are processed in the limbic system just like stress, positive stimulation of this brain region is a key to well-being. This has been used since ancient times, for example in aromatherapy. Scents are strongly coupled with memories and emotions. Thus, a certain scent can remind us of positive experiences, and we immediately relax. The scent of different herbs can trigger different body reactions. Rosemary scent can lower the cortisol level [6]. Citrus fragrances have anti-depressant effects. In Japan, the so-called Shinrin-Yoku is practised, a "forest bathing" in which an extensive relaxation effect can be achieved [7]. A large part of this is due to the scent of pine and cedrol, which lowers blood pressure and heart rate. The scent of these components is absorbed through the nose and activates olfactory receptors in the nasal mucosa. These are directly connected to the limbic system via the olfactory bulb. It causes a stimulation of the parasympathetic nervous system and a dampening of the sympathetic nervous system [8]. This is the reason for the deep emotional storage of olfactory experiences.

Recent research suggests that olfactory receptors are not only found in the nasal mucosa, but are also expressed by keratinocytes on the skin. Among the 396 different olfactory receptors discovered, 11 have been shown to be expressed ectopically in other tissues [9]. Only a few have been discovered on skin cells by now, e.g. keratinocytes. Here, the ORA2AT4 receptor, which can be activated by the scenting molecule sandalore, stands out in particular. It could be shown that the application of sandalore to the skin positively influences the differentiation and maturation of keratinocytes and can promote wound healing [10] or hair growth [11]. The activation of these receptors leads to a calcium influx, which results in a corresponding change in gene expression (Figure 1). A weakened calcium gradient in the epidermis caused by ageing or stress [12, 13] can thus be counteracted. This concept has long been used in aromatherapies such as Ayurvedic massages or other aromatic oil massages. Bitter substances, such as amarogentin, can bind to bitter taste receptors that are also expressed in the skin [14] and activate the similar signaling cascades. While the adenylate cyclase is activated during the activation of the olfactory receptors and the second messenger cAMP ensures the opening of specific calcium channels, phospholipase C is activated in the case of the bitter receptors. This releases the second messenger inositol 1,4,5-triphosphate (IP3), which activates the calcium channel IP3 receptor. Here, due to the release of calcium from the endoplasmic reticulum, there is an increase in the cytosolic calcium concentration [15].

Research into the effects of fragrance molecules on the skin is still in its infancy. It is important to remember that the application of a corresponding active ingredient is always a combination of direct effects and effects caused by the activation of the limbic system. This is because fragrance molecules are always absorbed through the nose and the smallest amounts are sufficient to activate the receptors. Here we look at the influence of a cosmetic active ingredient with a fragrance from *Myrothamnus flabellifolia* on the stress level of the mind, as well as its influence on the skin.

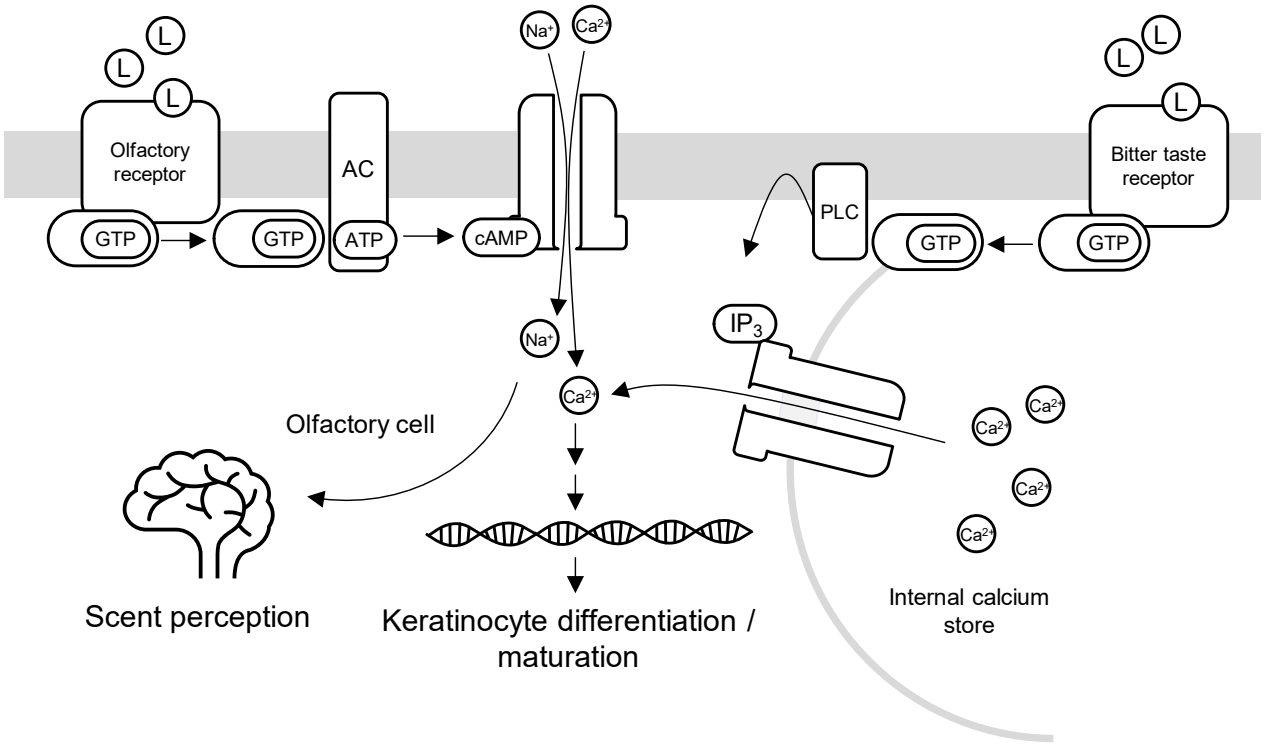

Figure 1: Activation of keratinocytes via olfactory and bitter taste receptors. Left: In cells of the olfactory mucosa, the binding of a scent molecule to the olfactory receptor leads to the activation of the associated G-protein. This activates the adenylate cyclase, which synthesises cAMP from ATP. cAMP activates cAMP-dependent sodium channels. This leads to an influx of sodium and subsequently also calcium. The cell depolarises and sends a signal to the brain. The information is translated into odors. Keratinocytes use the same system. Since these do not depolarise, there is only a calcium influx, which promotes expression of maturation and differentiation genes. **Right:** Bitter receptors work in a cAMP-independent way via phospholipase C: inositol triphosphate (IP3) is released from the membrane, which activates the IP3 receptor. This allows calcium ions to flow into the cytoplasm via internal calcium stores. The effect is the same as in the activation of olfactory receptors.

Material and methods

The Myrothamnus extract, (MYRAMAZE®-ESSENCE, RAHN AG, Switzerland; INCI: Caprylic/Capric Triglyceride, Myrothamnus Flabellifolia Leaf/Stem Extract) is the supercritical CO_2 extract of twigs/leaves of *Myrothamnus flabellifolia*. The resulting oily extract is diluted in neutral oil (CCT). Branches of the bush are harvested in wild pick in accordance with the South African Access and Benefit Sharing Regulations under the Nagoya Protocol. The project will support local communities and sustain the population of Myrothamnus bushes.

The essential oil fraction of the extract was determined by steam distillation (Ph. Eur. 2.8.12) and analysed for fragrance molecules by gas chromatography with a mass sensitive detector (GC-MSD). The fatty acid content was analysed according to DGF C-VI 10a and 11d.

Calcium influx was measured on normal human epidermal keratinocytes (NHEK). Intracellular calcium was detected by fluorescence labelling with Fluo-4 NW (Fluo-4 NW Calcium Assay Kit, Thermo Fisher Scientific). cAMP level was determined using a high sensitivity HTRF kit (cAMP GS Dynamic Kit, CisBio, France)

Fragrance characterisation was performed using standardised procedures (ISO 9235) with 12 trained individuals (10 female, 2 male). A scale from 0 to 10 was used for each identified scent aspect.

The *in vivo* studies were conducted in accordance with the Declaration of Helsinki of the World Medical Association. All study participants signed a written informed consent at the beginning of the study. To assess the effect of the product on stress levels, 25 study participants were stressed for 2.5 minutes with a series of standardised stress-inducing images [16]. After 5 minutes, saliva was collected to determine levels of the stress hormone cortisol and α-amylase. Afterwards, the study participants applied a cosmetic formulation with or without 3 % Myrothamnus extract (INCI: Water, Caprylic/Capric Triglyceride, Glyceryl Stearate Citrate, Cetearyl Alcohol, Phenoxyethanol, Sucrose Stearate, Carbomer, Caprylyl Glycol, Fragrance (Parfum), Xanthan Gum, Myrothamnus Flabellifolia Leaf/Stem Extract, Sodium Hydroxide). To keep the study double-blind, the formulations were perfumed with vanilla scent so that the intrinsic odour of the Myrothamnus extract could only be perceived subconsciously. A group of 10 test persons were allowed to smell the pure product. After 5 minutes, saliva was taken again to detect cortisol in a chemiluminescence assay (CLIA, IBL International, Hamburg, Germany) or to measure alpha-amylase via an enzyme kinetics assay (Roche Diagnostics, Mannheim, Germany). Immediately after the stress induction and after the treatment with the active ingredient, a Positive And Negative Affect Schedule (PANAS) questionnaire was filled out by the participants [17].

To measure skin parameters, the product was applied twice daily in a double-blind, placebo-controlled study. Measurements were taken 30 minutes and 24 hours after the first application without intermediate application. 25 subjects applied a placebo and 25 subjects applied the same formulation containing 1% Myrothamnus extract to the face. A third group of 25 participants applied 3% Myrothamnus extract to the face (INCI: Water, Caprylic/Capric Triglyceride, Glyceryl Stearate Citrate, Pentylene Glycol, Cetearyl Alcohol, Glycerin, Sodium Anisate, Sodium Levulinate, Xanthan Gum, Citric Acid, Myrothamnus Flabellifolia Leaf/Stem Extract). A hydration map was created by measuring 53 points on the face using corneometry (Corneometer MPA 580, Courage and Khazaka Electronic GmbH). TEWL was measured with a TEWAmeter (TEWAmeter TM 300, Courage and Khazaka Electronic GmbH, Germany). Wrinkles in the area of the crow's feet were measured using fringe projection (PRIMOS CR, Canfield Scientific, U.S.A.). Elasticity was determined using cutometry (Dual-Cutometer MPA 580, Courage and Khazaka Electronic GmbH, Germany).

Results

Extract properties: The supercritical CO_2 extraction of dry leaves/twigs of *Myrothamnus flabellifolia* resulted in an oily paste characterised by a content of more than 50 % of combined alpha-linolenic acid and linoleic acid. For use as a cosmetic active ingredient, the oily CO_2 extract was diluted in Caprylic / Capric Triglycerides (Myrothamnus extract).

The undiluted CO_2 extract contained 10 - 15 % essential oil. The composition was determined by GC-MSD and is shown in Table I. The fragrance molecules from two different batches of Myrothamnus harvest gave very similar results with the exception of Germacrene D, which was present at 24 % in one batch but not detected in the other (Table I). The main fragrance components of the essential oil are in consense with the fragrance characterisation of the Myrothamnus extract by a trained test panel. The fragrance was described on a scale from 0 to 10 (mean ± SD) as fir tree-like (7.25 ± 2.05), citrus-like (6.00 ± 1.81), herb-like (5.92 ± 2.39) with notes of acidity (3.75 ± 2.05) and fats (2.83 ± 1.47) [18]. An untrained test panel of 55 naïve women (n = 35) and men (n = 20) confirmed the fragrance profile, with the citrus scent being perceived more strongly by women, while the pine-like scents were perceived as dominant by men (not shown).

Compound	Sample 1 (%)	Sample 2 (%)	Scent
1,8-cineole	4.9	3.1	Eucalyptical
Alpha-copaene	2.0	3.6	Woody, spicy
Pinocarvone	20.5	18.7	Minty
Trans-pinocarveole	24.0	27.6	Herbal, woody, balsamic
Germacrene D	n.d.	24.1	woody
Trans-carveole	3.3	3.5	Caraway, solvent

Table I: Fragrance composition of the essential oil fraction. The fragrance characteristics of the main components correspond to the fragrance description of the expert panel. Only fragrances with a concentration of more than 3 % in the essential oil fraction are shown.

Effect of the cosmetic active ingredient on the mood: The study was designed to determine the effect of the fragrant molecules of Myrothamnus extract on the mood of study participants [18]. For this purpose, 25 test persons were stressed by looking at exciting pictures and the stress hormone level in their saliva was determined. This was determined again 10 minutes after applying an emulsion with 3 % Myrothamnus extract to the face. The same formulation without active ingredient served as a placebo. The scent of the Myrothamnus extract was masked with a vanilla perfume so that the scent of the extract could only be perceived subconsciously. Some of the test persons were presented with the pure Myrothamnus extract to smell. The analysis showed a significant decrease of the stress hormone cortisol by 6.8 % in the saliva after applying the emulsion with 3 % Myrothamnus extract to the face (Figure 2). The placebo had no effect. Smelling the Myrothamnus extract directly lowered the salivary cortisol level by 12.4 %, but only with limited significance. The alpha-amylase, which is also released under stress, was reduced by 12.4 % with limited significance by use of the emulsion. Here, too, there was a stronger reduction when smelling the active substance directly (23.5 %). These data are corroborated by the analysis of brain waves, especially the proportion of alpha waves. While applying the emulsion with 3% active ingredient slightly increased the proportion of alpha waves compared to the placebo value, the proportion was significantly greater when smelling the active ingredient. Since no significance could be calculated due to the measurement arrangement, these data are not shown. The questioning of the subjects' state of mind after application of the cosmetic formulations to the face showed a significant brightening of mood compared to placebo in the positive expressions of a PANAS questionnaire (not shown).

Figure 2: Decrease of stress hormone level after application of Myrothamnus extract. Both, cortisol and alpha amylase decreased 10 minutes after application of 3 % Myrothamnus extract in an emulsion (MYE 3 %) or after smelling of the pure active ingredient (MYE 100 %). N = 25 (placebo and MYE 3 %); n = 10 (MYE 100 %): Paired Student's t-test. * = $p < 0.05$.

Calcium mobilisation: Recent research has confirmed the presence of olfactory and bitter receptors on keratinocytes [14, 19]. Thus, an effect can be assumed not only through the absorption of scent molecules via the nose, but also through the activation of the corresponding receptors on the skin. Calcium mobilisation in keratinocytes can provide an indirect indication of the activation of these receptors. Indeed, Myrothamnus extract with increasing concentration can significantly increase calcium influx into the cytoplasm by 3.8-fold at 0.1% (Figure 3A). In contrast, the cAMP level in the cells was not increased (Figure 3B).

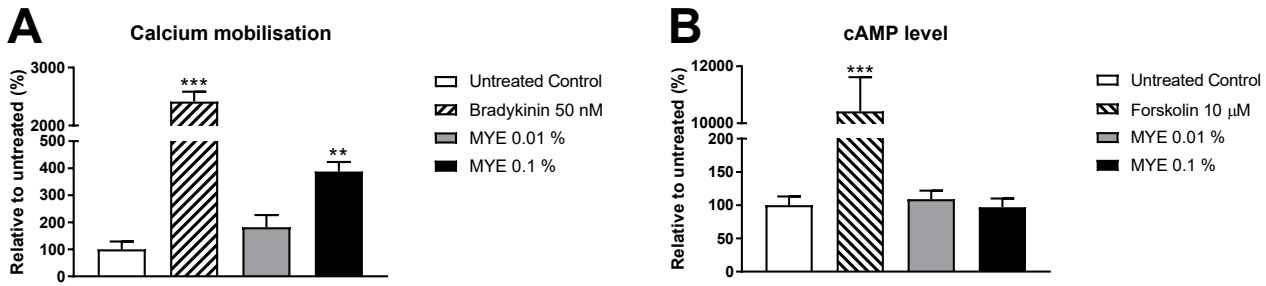

Figure 3: Calcium mobilization in keratinocytes. Increasing concentration of Myrothamnus extract (MYE) increase calcium concentration in the cytoplasm of NHEK cells (A). The cAMP level did not change (B). Bradykinin and forskolin served as positive controls. N = 3 for each condition. Unpaired Student's t-test. ** = $p < 0.01$; ***= $p < 0.001$.

Skin effects: To investigate the effect on human skin, a study was conducted with a total of 75 study participants. They applied either an emulsion without active substance (placebo), with 1 % or with 3 % Myrothamnus extract to the face. The study duration was 28 days, with measurements after 7 and 28 days. After 7 days, a significant reduction in transepidermal water loss (TEWL) was observed with both active ingredient concentrations (Figure 4A), levelling off at -11 % after 28 days ($p < 0.05$). After 7 days, the reduction was significantly higher than placebo ($p < 0.01$) with 3 % active ingredient use, and after 28 days both active ingredient concentrations were significantly higher than placebo. Placebo showed no effect on TEWL over the entire study period. For facial skin hydration, measurements were taken at 53 different sites

to create a hydration map. Total facial hydration increased significantly by 27% and 30% (Figure 4B) over placebo after 7 days and further increased to 46% and 51%, respectively, after 28 days ($p < 0.001$).

The visual representation of facial skin hydration (Figure 5) clearly showed a greater improvement when emulsions containing the active ingredient were applied compared to placebo. The detailed analysis of the facial areas showed a stronger hydration of particularly dry areas, such as the nasolabial fold. Hydration increased by 75 % when 1 % active ingredient was used and by 91 % when 3 % active ingredient was used. Placebo only gained 20 % in hydration in this area.

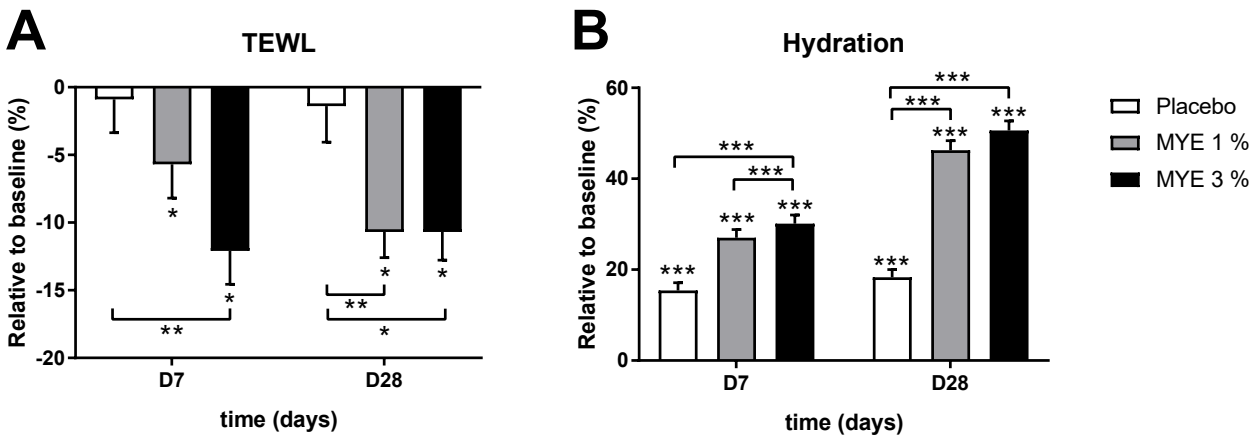

Figure 4: TEWL and skin hydration in the face. Application of 1 % and 3 % Myrothamnus extract (MYE) decreased TEWL (A) and increased skin hydration (B) after 7 or 28 days. N = 25 for each group. Paired / unpaired Student's t-test. * = $p < 0.05$; ** = $p < 0.01$; ***= $p < 0.001$.

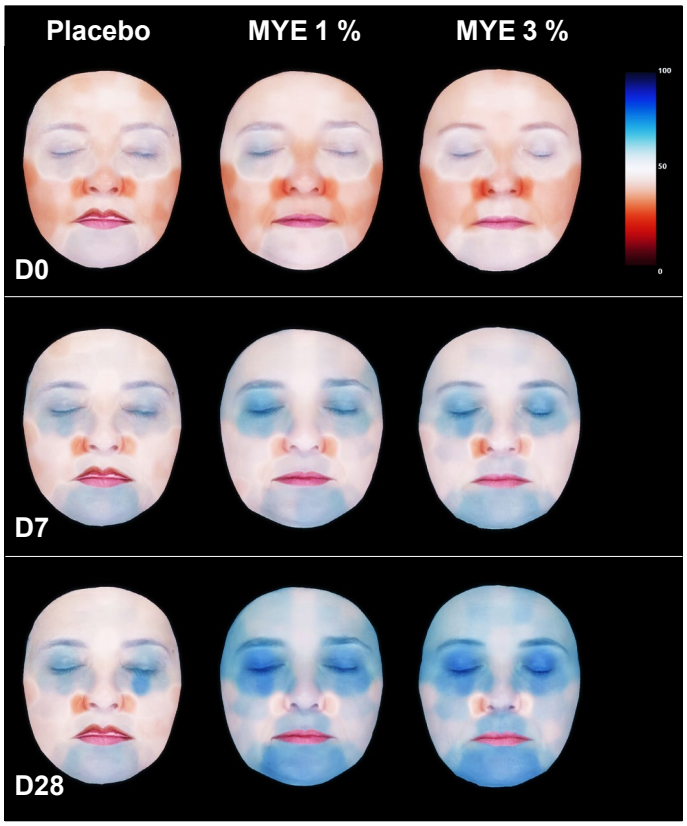

Figure 5: Hydration mapping of corneometer readings. Corneometer values of 53 spots on the face represent dry (red) or moist (blue) areas. Application of Myrothamnus extract (MYE) significantly increases moisture over placebo and baseline. Scale from 0 to 100. N = 25 for each group. Paired / unpaired Student's t-test (refer to Figure 3B).

The analysis of anti-ageing parameters (Figure 6) showed a significant reduction in wrinkle volume of 8% with 1% active ingredient and 10% with 3% active ingredient in the emulsion ($p < 0.05$). After 28 days, a reduction of 13 % was measured for both active ingredient concentrations, significant over placebo ($p < 0.05$). The placebo had no significant effect at all. Skin elasticity was improved equally by both active ingredient concentrations. After 7 days, elasticity increased significantly by 5 %, after 28 days by 10 %, significant over baseline and placebo ($p < 0.05$).

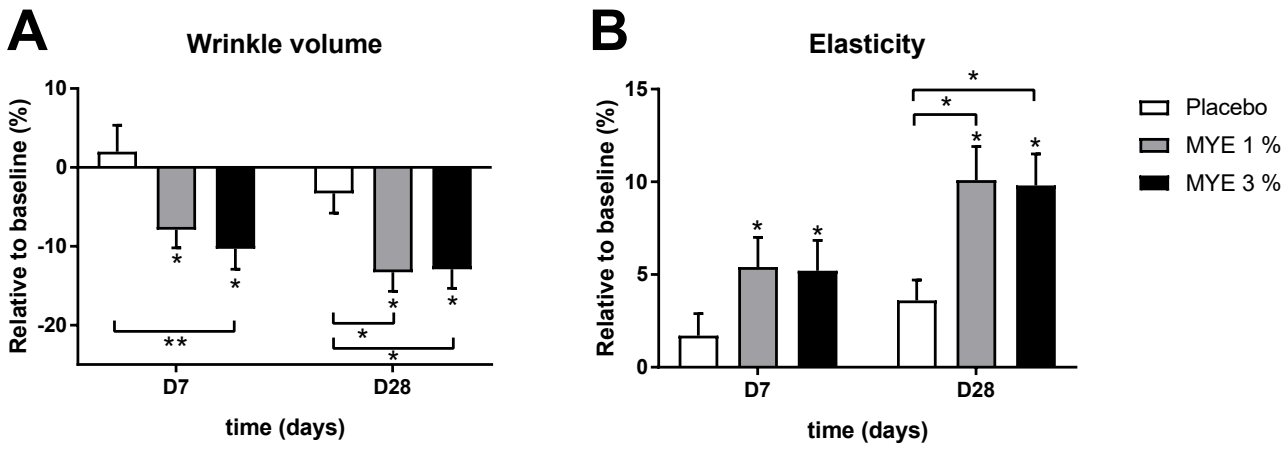

Figure 6: Anti-ageing parameters. Application of 1 % and 3 % Myrothmanus extract (MYE) decreased wrinkle volume (A) and increased skin elasticity (B) after 7 or 28 days. N = 25 for each group. Paired / unpaired Student's t-test. * = $p < 0.05$; ** = $p < 0.01$.

Discussion

By diluting the oily CO_2 extract of *Myrothamnus flabellifolia* in Caprylic/Capric Triglycerides, the proportion of the essential oil fraction was diluted to such an extent that the concentrations of the fragrance components were below the threshold of toxicological concern [20] (TTC Concept, Cramer Class III) and below the amount of declarable fragrance allergens in the EU, whereby none of the substances listed or planned in Annex III of the EU Cosmetics Act were found. Nevertheless, the extract was found to have an effect both through the absorption of the fragrance molecules via the nose and through the activation of receptors in the skin. With 3 % use, the active ingredient is still clearly perceptible via the nose in a basic cosmetic formulation. Activation of olfactory receptors in the olfactory mucosa is therefore evident. The stimulation of the test persons showed a mood-lifting effect and a stress-reducing effect. This is consistent with research on cedrol, a scent molecule that bears some resemblance to the scent of Myrothamnus extract, as it also has forest notes and smells like pine. Dayawansa et al. [8] were able to show that even very small amounts of 6.7 ppm cedrol absorbed through the olfactory mucosa reduced the heartbeat and blood pressure. The main fragrance components in Myrothamnus extract are present at a concentration of about 15 ppm when used at 3% in the final cosmetic, in total about 60 ppm of fragrance-active substances, i.e. 10 times more than the cedrol concentration investigated Dayawansa et al. The similar effect therefore seems plausible. A similar activity by the vanilla scent, which was used to cover the Myrothamnus scent, can be excluded as the placebo did not provoke any reaction. Neither mood parameters nor stress hormone levels changed due to the vanilla scent.

The stimulation of receptors that bind scent molecules was only measured indirectly on keratinocytes. Since both olfactory and bitter receptors cause an influx of calcium when a ligand is bound, this was measured as the first parameter. In fact, there was a significant calcium influx when Myrothamnus extract was applied. In principle, there are two classes of calcium channels: those that bring calcium from the extracellular area into the cytoplasm and those that serve internal calcium stores.

Since there was no increase in the cAMP level, we conclude that an adenylate cyclase-independent calcium channel was activated. Here, the IP3 receptor, which releases calcium from the endoplasmic reticulum, comes into question. Like olfactory receptors, bitter receptors are g-protein coupled receptors (GPCRs), so they can bind similar substance classes. Thus, it is likely that the fragrances from Myrothamnus extract can bind to bitter receptors as well. It is known that certain germacrene derivatives bind to the human bitter receptor hTAS2r4 [21]. However, based on the experiments presented, we cannot exclude the possibility that other calcium channels are activated in keratinocytes. One candidate here would be TRPM8, as this can be activated by thymol, for example, a fragrant substance of thyme, very similar to carveol, which is also present in Myrothamnus extract. However, thymol is an aromatic compound and carveol is only present in very small amounts (approx. 3 %) in the CO_2 extract. However, cross-activation cannot be ruled out either.

The application of Myrothamnus extract to the skin resulted in largely concentration-dependent effects that were already significantly above baseline and placebo after one week. The increase in skin hydration may have been caused by the stimulation of keratinocytes and an associated improvement in epidermal function. The fragrant components of the essential oil portion of the extract may be the active molecules here. The reduction in TEWL can also be interpreted as an expression of improved epidermal function. Here, the proportion of fatty acids as active molecules probably also plays a role, as a reduced TEWL could already be measured 30 minutes after application of the emulsions with active ingredient content, which was not the case with placebo (not shown). Thus, the incorporation of fatty acids from *Myrothamnus flabellifolia* into the skin barrier may directly depress TEWL. Skin ageing parameters such as wrinkles and elasticity were also investigated. While a measurable wrinkle volume reduction can already be achieved by increasing skin hydration, elasticity is a dermal parameter. The extent to which scent molecules also have an effect on fibroblasts has not yet been sufficiently researched. There are already reports that fibroblasts also express olfactory receptors [22]. However, our own experiments could not show calcium influx when fibroblasts were stimulated with the Myrothamnus extract (not shown). However, it could be shown that the fibroblasts relax

and lead to a greater flexibility of the matrix in a collagen contraction assay (not shown). Since Myrothamnus extract is a complex plant extract, synergistic effects of the plant metabolome may be the cause of the effect.

Conclusion

Myrothamnus extract is the supercritical CO_2 extract of the twigs and leaves of the resurrection bush *Myrothamnus flabellifolia* diluted in neutral oil. We determined the fraction of the essential oil and its composition. *In vivo*, it can stimulate olfactory receptors in the nose and relax the mood of study participants at concentrations of 3% active ingredient in an emulsion, even though the scent was not consciously perceived. *In vitro* experiments suggest activation of bitter taste receptors on keratinocytes, causing calcium influx but no increase in cAMP levels. The use of Myrothamnus extract strengthens the skin barrier, increases hydration and promotes skin elasticity. Myrothamnus extract is the first cosmetic active ingredient of its kind to combine the benefits of smelling with the nose and 'tasting' with the skin.

Acknowledgements

The methodology of the scent study is described here: [18]. The *in-vivo* skin study has been performed at PhDtrials, Portugal. Calcium influx and cAMP levels were studied at Qima, France.

Conflict of Interest Statement

Stefan Hettwer, Emina Besic Gyenge, Brigit Suter and Barbara Obermayer are employees of RAHN AG, Switzerland.

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