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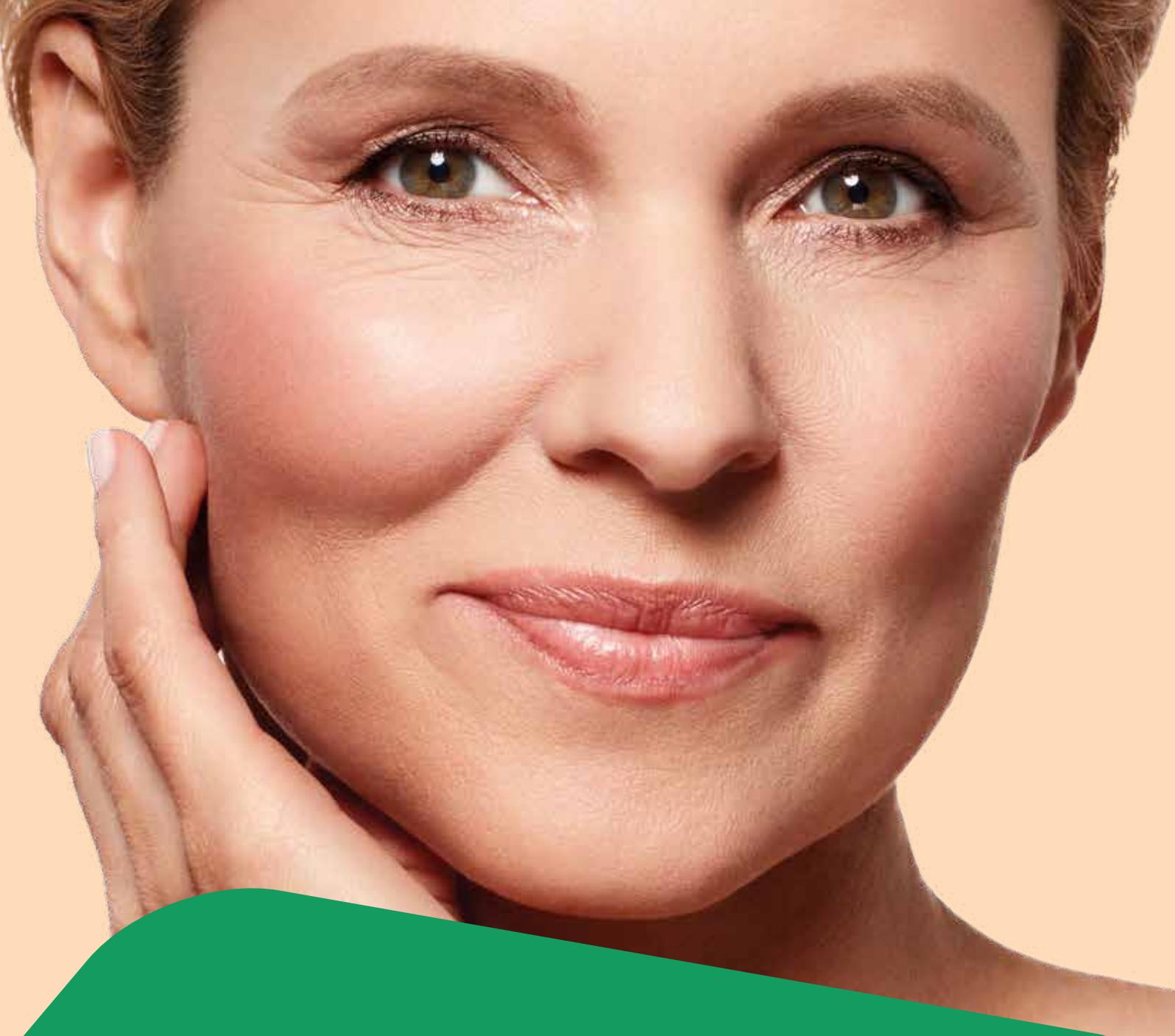
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## **The fascinating power of Nature to fight pollution: *Phyllanthus Emblica officinalis***

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# The fascinating power of Nature to fight pollution: *Phyllanthus Emblica officinalis*

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## Introduction

- Causes of air pollution are associated with e.g. combustion processes releasing thousands toxic pollutants like particulate matter and heavy metals (HM) notably from diesel exhausts. Upon exposure to a pollutant stress, reactive oxygen species (ROS) are generated, inflammatory cytokines and metallothionein overexpression (MT1H) are released.
- The associated consequences are visible signs of premature skin ageing, e.g. skin inflammation and/or enhanced skin pigmentation.
- Investigations (*in vitro* and *ex vivo*) were performed on an Indian goose berry tree extract well-known in the Ayurvedic medicine and obtained from a sustainable source: *Phyllanthus Emblica officinalis* (Emblica).

## Materials & Methods

### Cell culture

Monolayer cultures of Normal Human Epidermal Keratinocytes (NHEKs) were used for this study.

### ROS release from NHEKs

keratinocytes upon a challenge based on urban dust + UVA combination

Cultures were pre-incubated with the Emblica during 24h, then treated with a Emblica and urban dust (40µg/ml) for 24h and finally UVA-irradiated (5J/cm<sup>2</sup>) with UVA. Production of ROS are measured through fluorescence level emitted by the oxidized dye.

### Cytokine release from NHEKs keratinocytes upon urban dust challenge

Inflammation was induced by a treatment with urban dust (80µg/ml) for 24h. The cells were seeded 24h before the treatment Emblica, applied alone or associated to urban dust for the next 24h. The quantification of the inflammatory cytokines was performed using ELISA kits

### Ex vivo

Topical treatment with Emblica (0.5%) occurred on D0,D2,D3,D4 on a Caucasian man abdomoplasty. The explants were treated with a metals mixture and diesel particles (PM2.5) at 0.1%-on D4 for 24 hours. General morphology, MT-1H immunostaining and malondialdehyde (MDA) were studied.

## Results

### Emblica reduces ROS in NHEKs

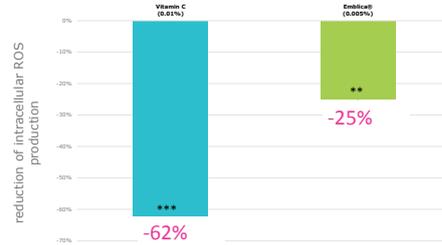


Figure 1: Intracellular ROS production induced by Urban Dust/UVA stress for Emblica (0.005%) and Vitamin C (0.01%). Results are shown as percentage relative to urban Dust/UVA condition fixed at 100%

### Emblica strongly reduces inflammatory cytokines and shows a soothing potential in NHEKs

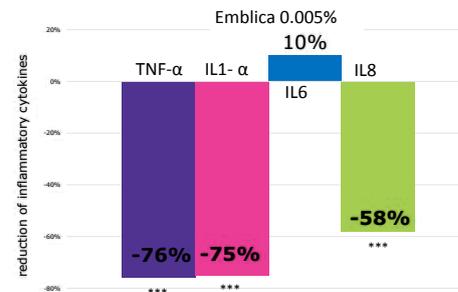


Figure 2: Release of inflammatory cytokines after a stress to urban dust treatment. Results presented as percentage relative to urban dust condition fixed at 100%. %; (statistics: Student test, \* p<0.05)

### Emblica protects against lipid peroxidation induced by heavy metals/diesel particles - ex vivo

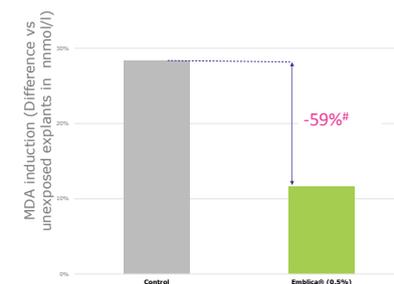


Figure 3: MDA induction after treatment with a pollutant mix (heavy metals and diesel particles (PM2.5)) (Difference vs. unexposed explants in nmol/l) for the control and Emblica (0.5%). # p<0.1. (Student t-test).

### Emblica decreases MT1H expression and shows a preventive anti-pollution effect - ex vivo

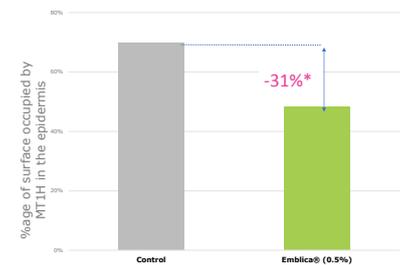


Figure 4: Surface occupied by MT1H in the epidermis of skin explants treated with the pollutant mix, for the control (stressed explant) and for the explant stressed with the pollutant mix and treated with Emblica (0.5%), (Student t-test, \* p<0.05)

### Emblica visibly reduces skin explant alterations after exposure to HM/diesel particles (PM2.5)

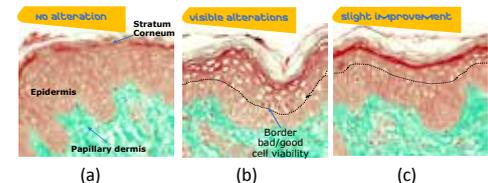
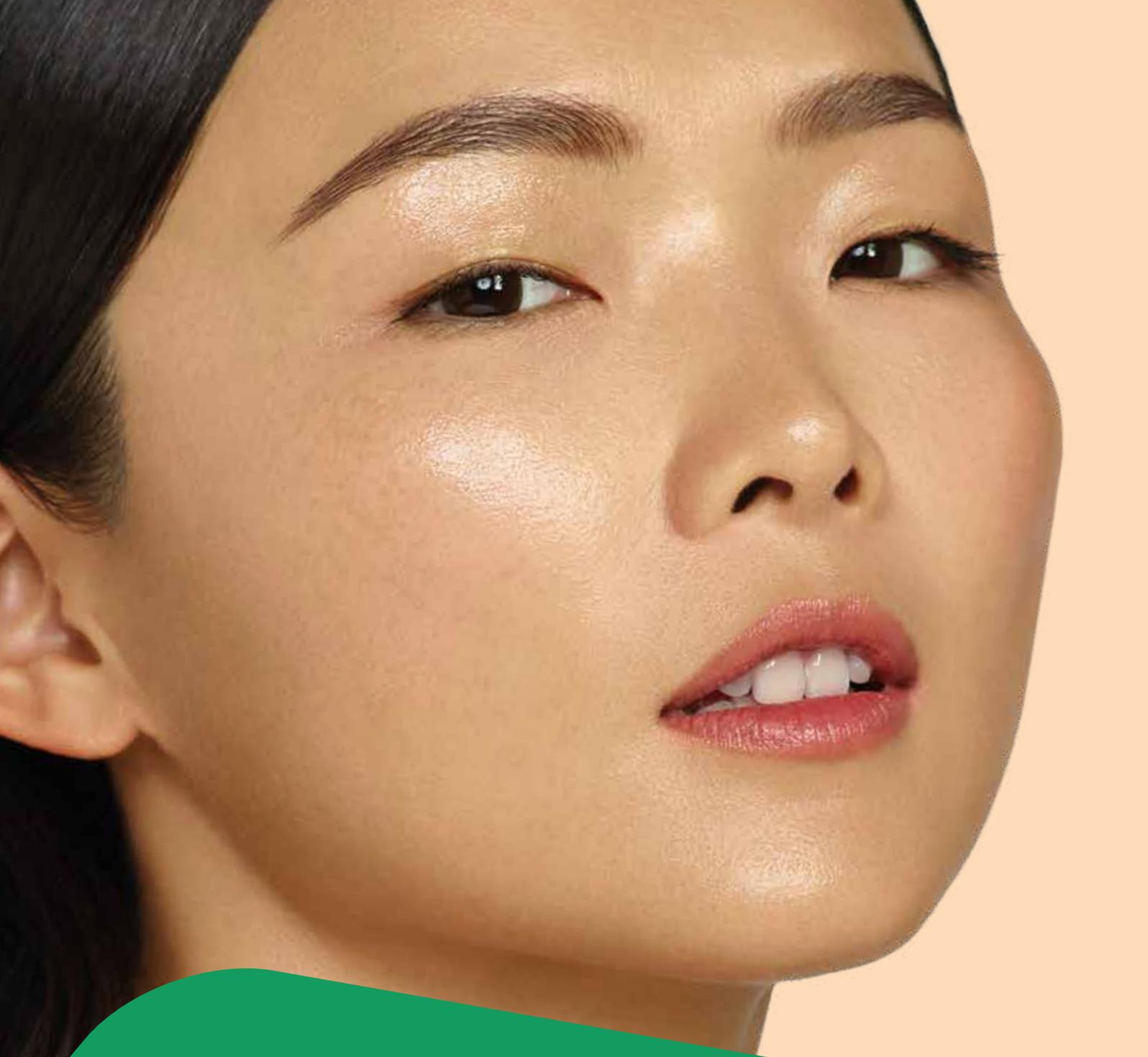


Figure 5: Explant morphology on Day 5. (a) untreated explant, (b) control explant stressed with the pollutant mix, (c) explant stressed with the pollutant mix and treated with Emblica (0.5%)

## Conclusion

- The results show a strong anti-pollution effect of Emblica in both models for all different parameters investigated in comparison to the control and the references.
- After topical application of Emblica, multiple biochemical parameters involved in different mechanisms of action could be positively impacted upon exposure to a pollution stress.
- These results highlight that *Phyllanthus Emblica officinalis* offers a simple solution to counterbalance human excesses arising from e.g. industrialization.



## **Inhibitory Effects of *Phyllanthus emblica* Tannins on Melanin Synthesis**

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## Inhibitory Effects of *Phyllanthus emblica* Tannins on Melanin Synthesis\*

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**KEY WORDS:** *Phyllanthus emblica*, melanin inhibitor, tannins, tyrosinase, peroxidase, iron chelator, individual typology angle, antifreckle

**ABSTRACT:** A standardized extract of *Phyllanthus emblica* (*Syn. Emblica officinalis*) fruits has been shown to be a safe and effective skin lightener for normal and hyperpigmented skin as demonstrated by several pilot clinical studies. This report shows melanin inhibitory activity for low molecular-weight hydrolyzable tannins.

Pigmentation of the skin because of synthesis and dispersion of melanin in the epidermis has a great significance in the cosmetic industry and society in general. It is the key physiological defense against sun-induced damage such as sunburn, photoaging and photocarcinogenesis.

Human skin color varies around the world. It ranges from a very dark brown in some African, Australian and Asian-Indian skin to a near pinkish-yellow among some northwest Europeans.

Complexion coloration in humans primarily is regulated by the amount and type of melanin synthesized by the epidermal melanocyte.<sup>1,2</sup> Melanocytes have specialized organelles termed *melanosomes* that contain several enzymes such as tyrosinase, tyrosinase-related protein-1 and tyrosinase-related protein-2, which catalyze the production of melanin.

Tyrosinase is the first and a rate-limiting step in melanogenesis.<sup>3</sup> However, additional and equally contributing

factors consist of efficient transfer of melanin from melanocytes to the neighboring keratinocytes and distribution and degradation of the transferred melanosomes by the recipient keratinocytes. The protease-activated receptor-2 (PAR-2) and unidentified surface lectins and glycoproteins facilitate this transfer process.<sup>4,5</sup> Skin-lightening agents (melanogenesis inhibitors) have been used widely to either lighten or even-tone the skin.<sup>6,7</sup> Preparations in the European market often are used to treat age spots and freckles or to obtain even-toning effects, whereas the Asian market uses them to change or modify skin color.

### *Phyllanthus emblica*

*Phyllanthus emblica* (*P. emblica*) has been used for more than 4,000 years for a variety of human ailments in ayurveda.<sup>8</sup> Its status ranges from insignificant in the western world to highly prized in tropical Asia. The antioxidant fraction of *P. emblica* (*Syn. Emblica officinalis*) fruits<sup>9</sup>, used in this investigation and referred to in the text as *P. emblica*, is different from other commercially

available extracts of *P. emblica* fruits. This is because it contains more than 50%, typically 65–75%, of key chemical components—low molecular-weight (<1,000) hydrolyzable tannins.

Previously, studies reported cascading antioxidant activity,<sup>9</sup> chelating activity,<sup>10</sup> antioxidant and matrix metalloprotease inhibitory activity<sup>11,12</sup> of a standardized extract of *P. emblica* fruit. No report has yet shown the melanin inhibitory activity of the low molecular-weight hydrolyzable tannins found in *P. emblica*; the goal of the present work is to investigate its effectiveness as a melanin inhibitor (in vitro and in vivo), to define its mode of melanin inhibitory activity, and to show its applicability as a cosmetic ingredient to lighten and even-tone normal and hyperpigmented skin color.

### Materials

Skin lightening agents, enzymes and reagents were obtained for comparison. Skin lightening agents included: *P. emblica*, hydroquinone, kojic acid, magnesium ascorbyl phosphate >95% pure<sup>b</sup>, *Glycyrrhiza glabra* (licorice) root extract<sup>c</sup> and ascorbyl-2-O- $\beta$ -D-Glucoside<sup>d</sup>.

Enzymes and reagents studied were: mushroom tyrosinase and horseradish peroxidase, potassium diphosphate, disodium EDTA, potassium hydroxide, L-DOPA, L-tyrosine, hydrogen peroxide and FeCl<sub>2</sub>. Human melanocytes from moderately pigmented donor

\*A part of this work was presented at the Society of Cosmetic Chemists (SCC) Annual Scientific Meeting held in May 2005, Las Vegas, USA.

<sup>b</sup>*Emblica* is a product of EMD Chemicals/Merck KGaA.

<sup>b</sup>RonaCare MAP (INCI: Magnesium ascorbyl phosphate) is a product of EMD Chemicals/Merck KGaA.

<sup>c</sup>Licorice PTH (INCI: Glycyrrhiza glabra (licorice) root extract) is a product of Barnett Products.

and human keratinocytes also were purchased.

## Methods

**In vitro melanin inhibitory activity:** Keratinocyte and melanocyte co-cultures were selected for this study. They were used at a 1:3 ratio in the fully supplemented 254 medium<sup>8</sup> in 6 well plates, at 30,000/96,000 cells/well. Cells were grown for 4 days for the pilot experiment and for 11 days for the subsequent tests with 2 growth medium changes. At the end of these tests, cells were counted and lysed with cellytic mammalian cell lysis/extraction reagent. Melanin content was determined spectrophotometrically at 405 nm. Cell cultures were monitored using an inverted microscope.

**In vivo skin lightening study (normal skin):** Healthy male and female subjects were used for the study. Subjects having history of sensitivity to facial skin care products, visible skin disease, or who were pregnant or lactating were excluded from the study. The study was conducted comparing baseline (pretreatment) to final (posttreatment) results of the upper left and right arms. For 9 weeks, each subject was given two products incorporating identical formulation ingredients, one with *P. emblica* (Formula 1) for the upper left arm and the other one with a different material (Formula 2) as a positive control for the right upper arm.

The panelists were instructed to apply approximately 0.5mL of the test materials twice daily. The selected test application sites were photographed before the study and after 3, 6 and 9 weeks of treatment. In order to determine the effect of the base formulation, if any, an additional study was conducted comparing the results of the product with *P. emblica* vs. a placebo without *P. emblica*. The subjects were instructed to record the date and time of each use of the test materials and asked to refrain from excessive sun exposure during the course of the study.

Rigid control of photographic tech-

nique, from the aspects of lighting, distance, angles and camera/film settings and specifications, and subsequent development of photographs were followed. Skin color measurements were performed using a tristimulus instrument<sup>d</sup>. Three consecutive readings of the test site were taken and the mean was used as the data point. The Repeated Measures Analysis of Variance and t-Test (dependant) were performed via computer.<sup>e</sup>

**ITA measurement:** The measurement

procedure for determining Individual Typology Angle (ITA) was performed in L\* a\* b\* mode following the European Expert Group on Efficacy Measurement of Cosmetics and Other Topical Products (EEMCO) recommendations.<sup>13</sup> ITA value is calculated from the mean L\* and b\* colorimetric values<sup>13,14</sup> using the formula:

Eq. 1

$$\text{ITA Degree} = \left[ \text{Arc Tangent} \left( \frac{L^* - 50}{b^*} \right) \right] \times 180/3.1416$$

### Formula 1. Skin-lightening Lotion with 2% *P. emblica*

A. Water ( <i>aqua</i> )	54.73% w/w
Disodium EDTA (Disodium EDTA, Universal Preserv-AChem)	0.05
Propylene glycol (Propylene Glycol, Lyondell)	5.00
B. Xantham gum (Vanzan NF, Vanderbilt)	0.25
Magnesium aluminum stearate (Veegum Ultra granules, Vanderbilt)	0.40
C. Cetearyl alcohol (and) cetearyl glucoside (Montanov 68, Seppic)	7.00
Apricot kernel oil (Lipovol P, Lipo)	10.00
Octyl stearate (Cetiol 868, Cognis)	3.00
Dimethicone (Dow Corning 200 Fluid 10cst, Dow Corning)	6.00
D. Water ( <i>aqua</i> )	10.00
<i>Phyllanthus emblica</i> fruit extract (Emblica, Rona)	2.00
E. Triethanolamine (TEA 99%/Union Carbide)	0.32
F. Phenoxyethanol (and) isopropylparaben (and) isobutylparaben (and) butylparaben (Liquapar PE, ISP)	1.00
G. Fragrance ( <i>parfum</i> ) (AB0013, Belmay)	0.25

**Procedure:** Disperse B in A and heat to 70–75°C. Separately combine C and heat to 70–75°C. Add C to AB while stirring. Homogenize until mixture cools to 60°C. At 30°C add D. Adjust pH with E to 4.0–5.0. Add F. Add G. Mix until uniform.

### Formula 2. Skin-lightening Lotion with 2% Hydroquinone

A. Water ( <i>aqua</i> )	33.93% w/w
Disodium EDTA (Disodium EDTA, Universal Preserv-AChem)	0.05
Propylene glycol (Propylene Glycol, Lyondell)	5.00
B. Xantham gum (Vanzan NF, Vanderbilt)	0.25
Magnesium aluminum stearate (Veegum Ultra granules, Vanderbilt)	0.40
C. Cetearyl alcohol (and) cetearyl glucoside (Montanov 68, Seppic)	7.00
Apricot kernel oil (Lipovol P, Lipo)	10.00
Octyl stearate (Cetiol 868, Cognis)	3.00
Dimethicone (Dow Corning 200 Fluid 10cst, Dow Corning)	6.00
D. Water ( <i>aqua</i> )	30.00
Hydroquinone (Hydroquinone, Universal Preserv-AChem)	2.00
E. Triethanolamine (TEA 99%, Union Carbide)	0.12
F. Phenoxyethanol (and) isopropylparaben (and) isobutylparaben (and) butylparaben (Liquapar PE, ISP)	1.00
G. Fragrance ( <i>parfum</i> ) (AB0013, Belmay)	0.25

**Procedure:** Disperse B in A and heat to 70–75°C. Separately combine C and heat to 70–75°C. Add C to AB while stirring. Homogenize until mixture cools to 60°C. At 30°C add phase C. Adjust pH with E to 4.5–5.0. Add F. Add G. Mix until uniform.

<sup>d</sup>The Chroma Meter CR-300 or the SmartProbe 400 can be used to measure skin color. The Chroma Meter CR-300 is a device of Konica Minolta, and the SmartProbe 400 is a device of IMS inc.

<sup>e</sup>ThinkPoint Software, v.5.01, for Windows was used.

Where  $L^*$  value is the lightness value,  $a^*$  is the color value in the red-green axis, and  $b^*$  is the color value in the blue-yellow axis. The higher ITA degree indicates the lightening of the skin.  $\Delta E$  of ITA degree was calculated by subtracting the average ITA degree of the treated site from that of the average baseline (day 1 of the study). Statistical significance of the data was determined by student t-Test.

Six separate clinical studies were performed to determine the potential of *P. emblica* as a skin lightener:

- **Study 1:** Comparison of 2% *P. emblica* vs. 2% hydroquinone (13 Asians);
- **Study 2:** Comparison of 2% *P. emblica* vs. 2% hydroquinone (13 Hispanics);
- **Study 3:** Comparison of 1% *P. emblica* vs. 3% magnesium ascorbyl phosphate (MAP, 16 Asians);
- **Study 4:** Comparison of 1% *P. emblica* vs. 2% kojic acid (16 African-Americans);
- **Study 5:** Comparison of 1% *P. emblica* vs. 0.1% *Glycyrrhiza glabra* (licorice) root extract (15 Hispanics); and
- **Study 6:** Comparison of 1% *P. emblica* vs. 3% ascorbyl glucoside (14 Hispanics).

Human volunteers with skin Types III and IV (as defined by the Fitzpatrick Photo-type Scale) were selected for all studies except study 4, in which skin types IV to VI were used.

**In vivo skin lightening study (hyperpigmented skin):** Healthy female subjects were used for the study. Subjects having history of sensitivity to facial skin care products, visible skin disease, or who were pregnant or lactating were excluded from the study. The study was conducted comparing baseline pretreatment to final posttreatment results of the selected test sites. The length of the study was 8 weeks. Each subject was given one lotion containing 1% *P. emblica*. The panelists, consisting of 19 Caucasian females with significant freckles, were instructed to apply the test material twice daily to the entire face. The selected test application site was photographed before the start of the study and then repeated after 4 and

8 weeks. Rigid control of photographic technique, as previously described, was followed. Skin color measurements were performed using a tristimulus instrument<sup>f</sup>. Repeated Measures Analysis of Variance was used to determine if any significant differences were observed in the mean Individual Typology Angle (ITA degree). Statistical significance of the data was determined by student t-Test.

### ***P. emblica* Melanin Inhibitory Action**

**Tyrosinase +  $H_2O_2$  with tyrosine or L-DOPA as substrate:**<sup>15, 16</sup> Tyrosinase catalyzes oxidation of tyrosine to L-DOPA, and also L-DOPA to DOPachrome, in the presence of  $H_2O_2$ , ultimately to formation of melanin in human skin. A mixture of substrate—1 mL, 6.3 mM for L-DOPA or 1.3 mM for tyrosine; hydrogen peroxide, 1 mL, 5 mM; and phosphate buffer, 2 mL, pH 7.4—was incubated for 10 min at 25°C. Then, 2 mL of this mixture was added to 1 mL containing 120 units of mushroom tyrosinase, the lightening ingredient and a buffer solution. After 15 min of reaction time, DOPachrome was formed having a characteristic absorption band at 475 nm. The spectra were recorded between 300–700 nm, and the optical density at 475 nm (due to DOPA-chrome) was measured.

**Peroxidase +  $H_2O_2$  with L-DOPA as a substrate:**<sup>15, 16</sup> This method is similar to that described above, except the enzyme used was horseradish peroxidase. Thus, a mixture of L-DOPA (1 mL, 6.3 mM), hydrogen peroxide (1 mL,

5 mM), and phosphate buffer (2 mL, pH 7.4) was incubated for 10 min at 25°C. The remainder of the method was identical to that described above.

**$Fe^{2+}$ :EDTA +  $H_2O_2$  with L-DOPA as a substrate:**<sup>15, 16</sup> Again, this method is similar to that previously described, except the enzyme is replaced with  $Fe^{2+}$ EDTA. Thus, a mixture of L-DOPA (1 mL, 6.3 mM), hydrogen peroxide (1 mL, 5 mM) and phosphate buffer (2 mL, pH 7.4) was incubated for 10 min at 25°C. The remainder of the method was identical to that described previously.

### **In vitro Melanin Inhibitory Activity**

The co-culture experiment was validated by microscopic observation, which revealed the presence of both keratinocytes and melanocytes. The 4-day pilot study showed a moderate (17%) reduction in melanin inhibitory activity of *P. emblica* at 50  $\mu$ g/mL, and practically no activity at lower concentrations tested (10 and 20  $\mu$ g/mL). Hydroquinone was found to be cytotoxic at 5  $\mu$ g/mL. The incubation time was prolonged in order to determine whether this results in a better melanin inhibitory activity. The result has been confirmed by additional studies, which showed that the inhibitory activity of *P. emblica* at 50  $\mu$ g/mL is very similar to that of kojic acid (50  $\mu$ g/mL). An 11-day incubation resulted in a 41–47% inhibition of melanin production, which were adjusted to cell numbers (Figure 1). The results have a direct correlation to the number of melanocytes present in the well. If the

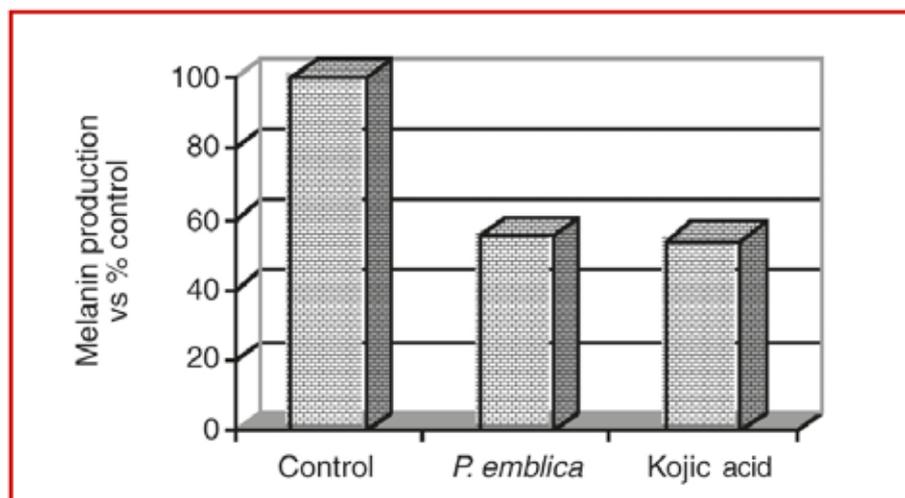


Figure 1: In-vitro melanin inhibitory activity of *P. emblica* vs. kojic acid

<sup>f</sup>The Chroma Meter CR-321 is a device from Konica Minolta.

cell numbers after the completion of the study are not taken into account, results could be due to cytotoxicity and not due to melanin inhibitory activity.

### In vivo Skin Lightening Activity: Normal Skin

Each comparative study consists of two products with identical formulation ingredients (one containing *P. emblica* and the other with a different material, positive control). No attempt was made to optimize these formulations. The results of these clinical studies are summarized in Figure 2.

The purpose of carrying out six clinical studies was to determine the skin lightening effect of *P. emblica* in comparison with well-known and

well-documented skin lighteners. In all six studies, *P. emblica*-containing product showed a significant increase in ITA degree (meaning skin lightening)<sup>13,14</sup> versus the corresponding baselines. Studies 1 and 2 clearly showed that 2% *P. emblica* has a comparable skin lightening effect to that of 2% hydroquinone in Asian as well as Hispanic subjects. Study 3 showed that 1% *P. emblica* was at least three times better in skin-lightening efficacy on a percentage-active basis over the 3% MAP product. Studies 4 and 5 showed 1% *P. emblica* was comparable to 2% kojic acid and 3% ascorbyl glucoside, respectively. Study 6 showed 1% *P. emblica* was comparable to 0.1% *Glycyrrhiza glabra* (licorice) root extract (0.1%).

### In vivo Skin Lightening Activity: Hyperpigmented Skin

*P. emblica*-containing product showed a significant increase in ITA degree<sup>13,14</sup> and L value versus the corresponding baselines, especially after 8 weeks. After the 8 weeks of treatment, reduction in freckle spots was seen in 17 out of 19 subjects, which is statistically significant ( $p < 0.005$ ). The result of this clinical study is summarized in Figure 3.

### Mechanism of Melanin Inhibitory Action of *P. emblica*

*P. emblica* has a unique mechanism of action because it acts at several different sites in the melanogenesis pathway. It inhibits two enzymatic pathways catalyzed by tyrosinase—tyrosine to L-DOPA and L-DOPA to DOPACHrome, and peroxidase/H<sub>2</sub>O<sub>2</sub> DOPA to DOPACHrome activity. It also inhibits Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub>-induced melanogenesis by quenching Fe<sup>2+</sup>. Chelating activity of *P. emblica* has been previously reported.<sup>10</sup> Based on the peroxidase inhibitory activity of *P. emblica* in the conversion of L-DOPA to DOPACHrome, it is quite conceivable to assume that *P. emblica* also can inhibit peroxidase/H<sub>2</sub>O<sub>2</sub>-induced conversion of dihydroxy indole (DHI) and dihydroxy indole carboxylic acid (DHIC) to melanin. Prota et al. demonstrated that peroxidase is much more effective than tyrosinase in catalyzing conversion of DHI and DHIC to melanin synthesis.<sup>17</sup>

It is reported<sup>16</sup> that ultraviolet radiation (UVR) is able to act directly on cells to bring about delayed increases in

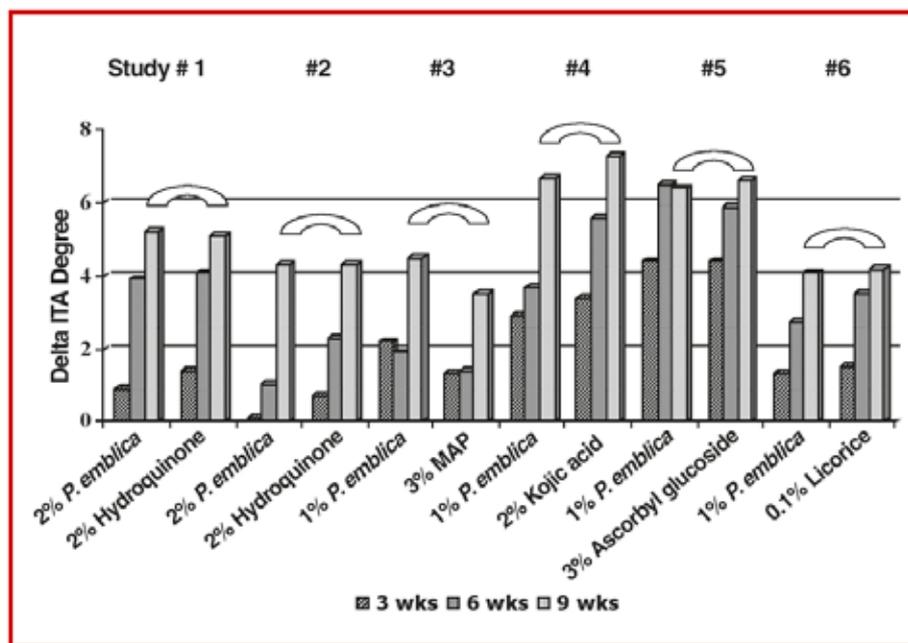


Figure 2: Results of clinical studies of *P. emblica* and other skin lighteners in lotion

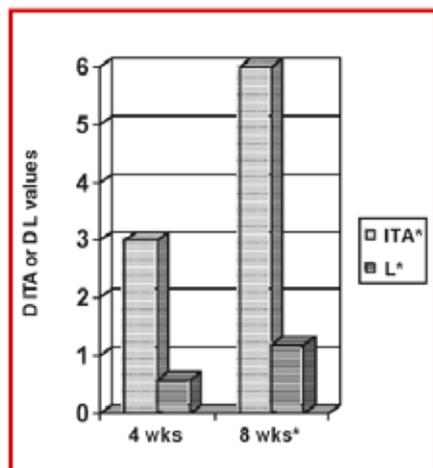


Figure 3: Results of anti-freckle clinical study with 1% *P. emblica* lotion

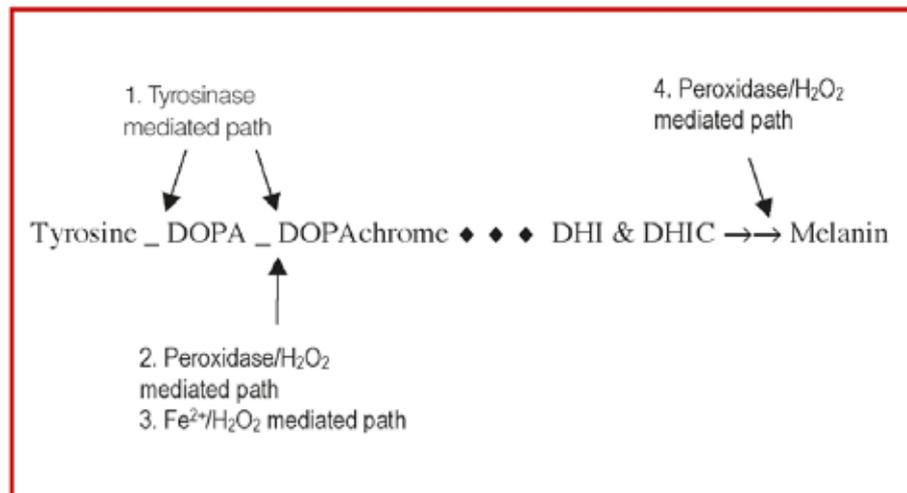


Figure 4: Sites of action of *P. emblica* in the melanogenesis pathway

melanogenesis. UVR also stimulates melanogenesis through a more rapid action that is not associated with an activation of tyrosinase. This effect could be mediated by the superoxide anion which, rather than activating tyrosinase, could act by serving as a substrate for the enzyme.<sup>18</sup> Since *P. emblica* has a strong superoxide anion inhibitory activity,<sup>11,12</sup> it is quite reasonable to assume that it inhibits melanogenesis by quenching superoxide anion radical as well.<sup>19-21</sup> All data suggests that melanin inhibitory activity of *P. emblica* is explained by inhibition of the major enzymes involved and also, inhibition of Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub>- and superoxide anion-induced melanogenesis. Figure 4 summarizes the mechanism of action of *P. emblica*.

## Conclusion

Melanogenesis is the process of production and subsequent distribution of melanin by melanocytes and is controlled by many factors. Skin lighteners can inhibit tyrosinase activity or block the chain reaction at various points of the melanogenesis pathway. Tyrosinase inhibition is the key mode of action in most commercial skin lighteners. *P. emblica* was found to inhibit two enzymatic pathways catalyzed by tyrosinase and inhibit Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub>-induced melanogenesis by the chelation of Fe<sup>2+</sup> and superoxide anion-induced melanogenesis by quenching superoxide anion. It has been reported mistakenly that *P. emblica* works by inhibiting  $\alpha$ -melanocyte stimulating hormone.<sup>22</sup>

*P. emblica* has been shown to be a safe<sup>22</sup> and effective skin lightener as demonstrated by several clinical studies. Increasing use of skin-lightening agents among global population has made it apparent that both the efficacy of a skin lightening formulation, as well as safety and mildness of the skin lightening ingredient, are equally important. Many current skin lighteners such as hydroquinone<sup>23,24</sup> and kojic acid<sup>25,26</sup> have inadequate safety<sup>27</sup> and stability profiles. The US Food and Drug administration (FDA) has recently proposed a ban on the sale of products containing hydroquinone.<sup>28</sup> The proposed ban

comes after mounting evidence linking the use of hydroquinone to potential health risks. The FDA is now describing hydroquinone as a possible carcinogen. Also, the FDA reports consumers have developed ochronosis after using hydroquinone products for as little as three months. Based on the toxicity problems of hydroquinone and kojic acid, it is quite reasonable to question the safety of arbutin (hydroquinone- $\beta$ -D-glucoside) and kojic acid dipalmitate for human use. Recently, a case of contact dermatitis due to arbutin has been reported.<sup>29</sup>

*P. emblica* also was shown in a recent unpublished clinical study to reduce the appearance of wrinkles and fine lines. A properly constituted *P. emblica*, such as the one described and tested, could provide a great value as a photoprotective agent in combination with sunscreens and an antiaging and skin-lightening agent. The effects and skin care benefits of *P. emblica* make it an ideal choice for a variety of cosmetic products targeting young and matured skin alike.

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## **Low Molecular Weight Tannins of *Phyllanthus emblica*: Anti-Aging Effects**

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# Low Molecular Weight Tannins of *Phyllanthus emblica*: Anti-Aging Effects\*

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Ultraviolet irradiation from the sun has deleterious effects on human skin, including sunburn, immune suppression, cancer and premature aging (photoaging). Sunburn and immune suppression occur acutely in response to excessive exposure to the sun, whereas skin cancer and photoaging result from accumulated damage caused by repeated exposures. Skin cancer, the most prevalent form of cancer in humans, typically occurs in skin that is photoaged.<sup>1</sup> Photoaged skin is characterized by wrinkles, uneven pigmentation, brown spots and a leathery appearance.<sup>2</sup> In contrast, chronologically aged skin that has been protected from the sun is thin and has reduced elasticity but is otherwise smooth and unblemished.<sup>3</sup>

The unifying pathogenic agents for these changes are UV-generated reactive oxygen species (ROS) that deplete and damage non-enzymatic and enzymatic antioxidant defense systems of the skin, and the release of matrix metalloproteases (MMPs) such as MMP-1 and MMP-3 that damage the extracellular matrix proteins.<sup>4</sup> Photoaging of the skin is a complex biological process affecting various layers of the skin with major damage seen in the connective tissue of the dermis. The dermis lies below the epidermis, and in conjunction with the basement membrane at the dermal-epidermal junction, provides mechanical support for the outer protective layers of the epidermis. Any damage to the dermal components is seen predominantly on the sun-exposed body areas, especially on the face.

This paper focuses on the major causes of the photoaging of skin and the use of low molecular weight tannins from *Phyllanthus emblica* fruits (Syn. *Embllica officinalis*) in reducing some of the causes of premature skin aging.

## Major UV-Induced Chemical and Biochemical Changes

**Generation of superoxide, singlet oxygen and hydrogen peroxide:** There is now ample evidence showing that reactive oxygen species (ROS), generated in vitro and in vivo after UVA and UVB irradiation, cause serious damage to skin.<sup>5</sup> Besides direct absorption of UVB photons by DNA and subsequent structural changes, generation of ROS following irradiation with UVA and UVB requires the absorption of photons by endogenous photosensitive molecules.

Recently, researchers<sup>6</sup> identified the epidermal UVA-absorbing chromophore transurocanic acid that quantitatively accounts for the action spectrum of photoaging. The excited photosensitizer subsequently reacts with oxygen resulting in the generation of ROS including superoxide anion and singlet oxygen.

Superoxide anion and singlet oxygen are also produced by neutrophils that are present in increased quantities in photo-damaged skin, and contribute to the overall pro-oxidant state. Superoxide dismutase (SOD) converts superoxide anion to hydrogen peroxide. Hydrogen peroxide is able to cross cell membranes easily and, in conjunction with Fe<sup>2+</sup>, generates a highly toxic hydroxyl radical. Both singlet oxygen and hydroxyl radical can initiate lipid peroxidation.

To counteract the harmful effects of ROS, the skin is equipped with antioxidant defense systems consisting of a variety of low molecular weight antioxidants (such as vitamin C and vitamin E) and antioxidant defense enzymes (such as superoxide dismutase, glutathione peroxidase and catalase) forming an "antioxidant network."

The antioxidant network is responsible for maintaining the equilibrium between pro-oxidants and antioxidants. However, the antioxidant defense can be overwhelmed by increased exposure to exogenous sources of ROS. Such a disturbance of the pro-oxidant/antioxidant balance may result in oxidative damage to lipids, proteins and DNA. A review of the protective effects of topical antioxidants in humans has recently been published.<sup>7</sup>

**Release of iron and copper:** Iron and copper play ambivalent roles in biology because they are required as cofactors for many biological reactions, even though their toxicity threatens cellular integrity.<sup>8</sup> In mammalian cells, the level of iron-storage protein is tightly controlled by the iron-regulatory protein-1 at the post-transcriptional level. This regulation prevents iron from acting as a catalyst in reactions between ROS and biomolecules.

Recently, it has been shown that both UVB and UVA can generate lipid peroxidation induced by iron.<sup>9,10</sup> The iron content is substantially elevated over basal levels in the skin of mice

## Key words

anti-aging, photoaging, antioxidant, reactive oxygen species, iron chelator, matrix metalloprotease, *Phyllanthus emblica*, tannins

## Abstract

The three major causes of premature photoaging of skin can be reduced by using low molecular weight tannins of *Phyllanthus emblica* fruit extract. Product description, standardization, stability and formulation guidelines are discussed.

\* Adapted from a paper presented by R.K. Chaudhuri at the Active Ingredients Conference – A Perspective on Naturals and their Actives, Paris, 2003

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exposed to UVB irradiation and in the sun-exposed skin of healthy individuals.<sup>11,12</sup> The underlying mechanism appears to be the UVB-induced formation of superoxide radical and its attack on ferritin, resulting in the release and mobilization of free iron.<sup>13</sup>

Brenneisen et al. have identified the iron-dependent Fenton reaction and lipid peroxidation as the central mechanisms underlying signal transduction of the UVB response.<sup>9</sup> Singlet oxygen and hydrogen peroxide are presently considered to be the most important reactive oxygen species generated intracellularly by UVA light promoting biological damage in exposed tissues via iron-catalyzed oxidative stress.<sup>14</sup>

Gutteridge et al. have found copper in sweat samples from the arm or trunk of athletes immediately after exercise.<sup>15</sup> Arm samples also contained much greater concentration of iron after exercise. It is also easy to see from these data how athletes following an intensive training might become anemic by loss of iron.

Unfortunately, skin does not have any defense against oxidative stress induced by free iron and copper. Application of metal chelators having chelating ability to occupy all the coordination sites in iron and copper may be a route to prevent or reduce oxidative damage to skin.<sup>16</sup> The iron-chelating agents have been shown as protectants against UV-radiation-induced free radical production.<sup>12,17</sup>

**Release of matrix-degrading MMPs:** Matrix metalloproteases (MMPs) are enzymes able to degrade most components of the extracellular matrix (ECM). Among these components are collagens, elastins, fibronectin and proteoglycans.

At this time more than 20 different MMPs have been identified and classified.<sup>18</sup> They show consistent sequence homology and in general share a pre-domain (which is a signal peptide for secretion), a pro-domain (important for maintaining latency) and a catalytic domain (with a highly conserved zinc-binding site). Based on sequence homology and substrate specificity, MMPs can be classified in the five groups: collagenase, gelatinases, stromelysins, membrane type, and others. This classification is somewhat arbitrary, because the true physiological substrates are a matter of debate.

The ECM proteins not only provide a supportive function for the development and organization of tissues, but also serve as a physical barrier to limit the migration of most normal cells away from their sites of origin. The ECM is not a homogeneous structure. It can include any of several classes of biomolecules, including the following: structural proteins, such as collagens and elastin; adhesion proteins, including fibronectins, laminins, and entactin; proteoglycans; and glycosaminoglycans. Further, the precise compositions of the ECM vary between tissues, and perhaps even in a cell state-specific manner. This complex mixture does not simply surround cells, hold them together, and provide an environment in which interesting events occur; it also directly or indirectly mediates a number of critical biological processes.

Several studies carried out by Scharffetter-Kochanek's group using dermal fibroblast cells show that both UVA and UVB cause a 4-fold to 5-fold increase in the production of MMP-1 and MMP-3.<sup>9,19,20</sup> In contrast, the synthesis of tissue inhibitory metalloprotease-1 (TIMP-1), the natural inhibitor of matrix metalloprotease, increases only marginally. This imbalance is one of the causes of severe connective tissue damage resulting in photoaging of the skin.

The damage caused by excessive MMP on the ECM proteins does not appear overnight, but results from the accumulation of successive molecular damages, especially in the case of overexposure to UV light. However, the

degradation of the ECM proteins has consequences for the skin. These consequences may be revealed in many ways depending on age, genetic predisposition, life-style and, of course, on the general health status of the individual.<sup>21</sup> Application of MMP inhibitors may be a route to prevent or minimize damage to ECM proteins.<sup>22</sup>

## Low Molecular Weight Tannins of *Phyllanthus emblica* Fruits

**A tannin-based *P. emblica* extract:** *P. emblica* is one of the important Ayurvedic (Science of Life) herbs in India, and has been used for thousands of years in a wide variety of human ailments. Its status ranges from insignificant in the western world to highly prized in tropical Asia. The fruits are selected, harvested and processed according to strict criteria to ensure a consistently high quality product.

A particular *P. emblica* extract<sup>4</sup>, protected by a U.S. patent<sup>23</sup> and other pending patents, is extracted from premium quality fruits using a water-based process. This extract is distinctly different from other commercially available extracts of *P. emblica* fruits because it is defined to the extent of well over 50% (typically, 60-75%) in terms of its key active components, which are low molecular weight hydrolyzable tannins. None of the other *P. emblica* extracts in the market compares to this tannin-based extract in terms of composition and consistency of composition, aqueous stability and color. In this article, we will refer to the product as a tannin-based *P. emblica* extract, or a TBPE extract, because this extract is based to such a large degree (60-75%) on these tannins.

**Tannins:** Tannins are chemically complex substances widely distributed in the plant kingdom and employed in medicine as astringents. They possess an abundance of polyphenols, have molecular masses in the range of 500-5000 Dalton, and display a diversity of structures that form the basis of their classification into two families, of which the hydrolyzable tannins are of interest in this article.

As its key ingredients, the patented TBPE extract contains four of these hydrolyzable tannins (Figure 1): Emblicanin A, Emblicanin B, pedunculagin and punigluconin. In nature, Emblicanin A and Emblicanin B have only been found in *P. emblica* plants.<sup>24</sup>

**Standardization and stability:** The TBPE extract has been standardized<sup>25</sup> by using either high performance thin layer chromatography (HPTLC) or high performance liquid chromatography (HPLC).

It is stable in aqueous as well as in formulated products for well over two years. It is extremely photostable, which was determined by exposing a 1% aqueous solution and a formulated product of this extract to UVA and UVB irradiation separately for a period of four hours (about 8 minimum erythemal dose, MED). Photostability of the product was determined from the  $\lambda_{\max}$  value at 271 nm and normalized with respect to the time zero. A loss of only 3% was observed after irradiating the product for 2 hrs (4 MED). On the contrary, a vitamin E-containing formulated product (where the vitamin E was natural tocopherol and  $\lambda_{\max}$  was 263 nm) lost well over 70% in 2 hrs (4 MED).

The photostability of an active in a formulation under UV irradiation is tested by ultra-thin film transmission of light. An ultra-thin film of the formulation is prepared between glass or quartz slides as to allow a minimum of 90% light transmission at 600 nm. Formulations are subject to two difficulties: (i) light scattering by the emulsion (mostly multiple scattering) and

<sup>4</sup> *Emblica Antioxidant (INCI: Phyllanthus emblica fruit extract)* is a product of Rona, a division of EMD Chemicals, Inc., Hawthorne, New York, USA.

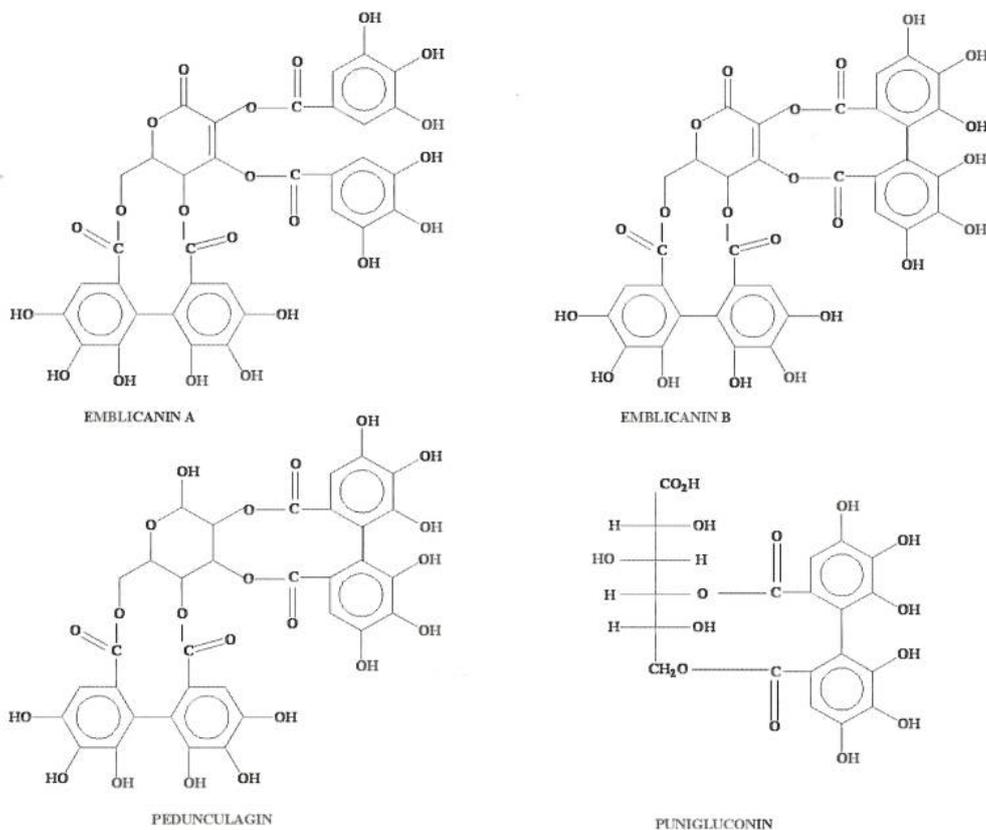


Figure 1. Hydrolyzable tannins of *Phyllanthus emblica* fruits

(ii) a nontransparent background over which to measure the UV absorption of the active. While the laws of light scattering are known, these effects are limited by measuring the properties of an ultrathin film of product. In this case, chromophore absorption becomes clearly visible above the strongly reduced light scattering background.

The UV photodegradation of an active can therefore be measured from its decrease in the characteristic UV absorption band, after correction for the light scattering background. This method requires a minimum concentration of active in order to provide a good signal-to-background ratio and is performed for identical formulation bases in order to compare actives.

TBPE extract has been found to have broad-spectrum antioxidant activity,<sup>26</sup> excellent iron- and copper-chelating ability and MMP-1 and MMP-3 inhibitory activity. These multifunctional attributes of TBPE extract are described in the following sections.

### Quenching of ROS

There are dozens of testing methods available for determining the ROS quenching ability of a substance. We are describing here results of three tests – superoxide anion, hydroxyl radical quenching and singlet oxygen – carried out for TBPE extract and a few commercially available antioxidants.

**Cell protection against superoxide damage:** A hypoxanthine-xanthine oxidase test evaluates cell protection against superoxide damage. About 90% protection of fibroblast cells against superoxide damage was observed using 20 µg/ml of TBPE extract.

This study uses a human skin fibroblast cell culture to determine the cell viability under superoxide anion [gener-

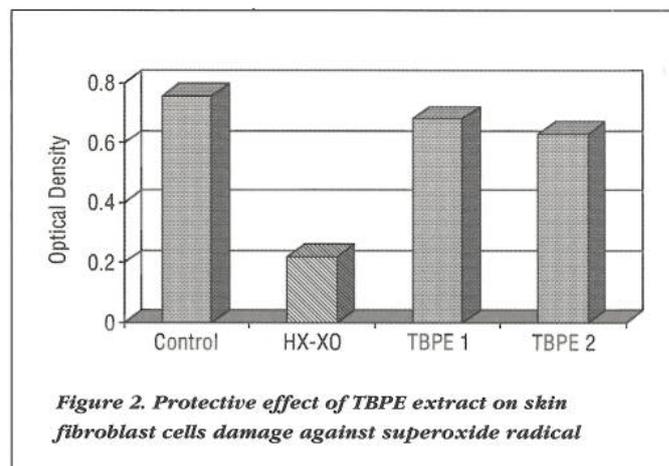
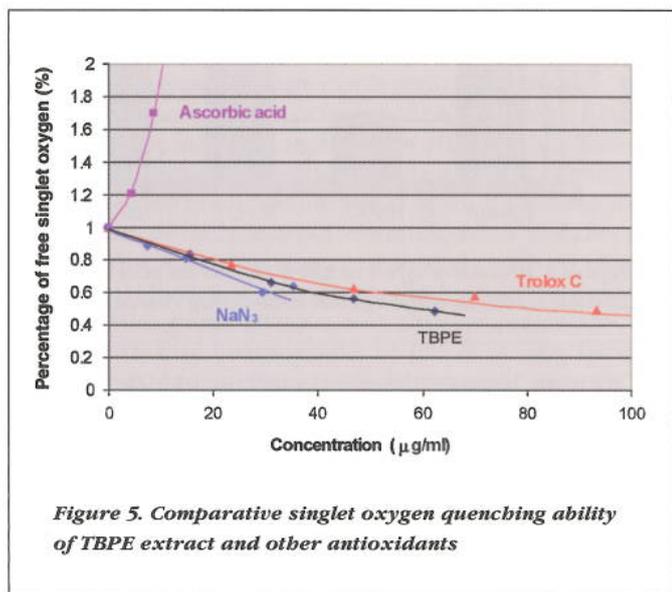
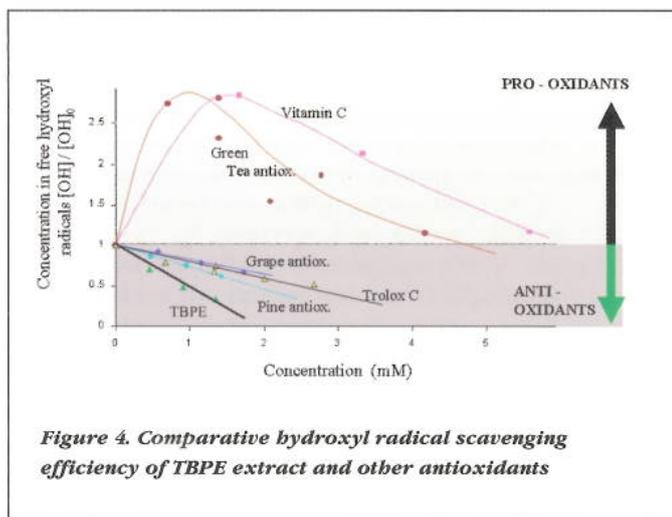
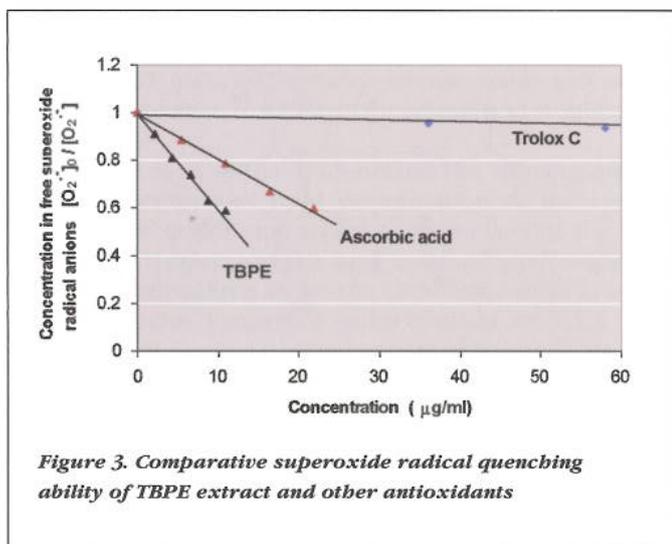


Figure 2. Protective effect of TBPE extract on skin fibroblast cells damage against superoxide radical

ated by using hypoxanthine and xanthine oxidase (HX-XO) system<sup>27</sup>] and the ability of TBPE extract to protect cells under these conditions. Two different batches (1 and 2) of TBPE extract were used for this study. Cell survival was determined with a colorimetric method<sup>28</sup> using 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT). The test is an indication of living cells, which is determined by measuring the optical density at 570 nm (Figure 2).

**Protection of dye against superoxide damage:** Another hypoxanthine-xanthine oxidase test evaluates protection of dye against superoxide damage. Superoxide anions are generated in an aqueous solution of hypoxanthine (100 µM) in the presence of EDTA (1 mM) by adding 0.014 units of xanthine oxidase freshly diluted in 100 µL of phosphate buffer (pH 7.4). The production of superoxide anions is determined through its reduction of NBT (nitroblue tetrazo-



lium) into nitroblue diformazan by monitoring the light absorption of NBT at 560 nm.<sup>29</sup> Spectrophotometric measurements are thus used to determine the antioxidant efficiency of various antioxidants. Efficient superoxide anion scavenging results in reduced NBT destruction and, thus, in a stronger light absorption at 560 nm.

Results of this study are given in Figure 3. IC<sub>50</sub> of the TBPE extract was found to be 12 µg/ml, compared to 26 µg/ml for

vitamin C and 360 µg/ml for a vitamin E water-soluble analog<sup>c</sup>.

**Hydroxyl radical quenching:** The deoxyribose test evaluates hydroxyl radical quenching. Results demonstrate that TBPE extract possesses the strongest hydroxyl radical scavenging ability, significantly better than pine antioxidant, grape antioxidant and a vitamin E product<sup>c</sup> with the alkyl chain substituted by a carboxylic group. Vitamin C and green tea extract have been shown to be pro-oxidant by this testing method, following the protocol developed by Halliwell et al.<sup>30</sup>

The hydroxyl radical scavenging efficiency of products was obtained at respective concentrations of 0.3 mM of FeCl<sub>3</sub>, 1.2 mM of EDTA, 33.6 mM of H<sub>2</sub>O<sub>2</sub>, 33.6 mM of deoxyribose in pH 7.4 phosphate buffer (20 mM) and 0.2-10 mM of chelator. The amount of hydroxyl radicals was determined from the deoxyribose test by using 1% w/v of thiobarbituric acid (TBA) and 2.8% w/v of trichloroacetic acid (TCA). Results are summarized in Figure 4.

**Singlet oxygen quenching:** Singlet oxygen quenching can be evaluated by photooxidation of a sensitizer. Results show that TBPE extract (IC<sub>50</sub> 61 µg/ml) is an excellent singlet oxygen quencher and is superior to the vitamin E water-soluble analog<sup>c</sup> (IC<sub>50</sub> 84 µg/ml). In this test, vitamin C was found to be a strong enhancer of singlet oxygen (pro-oxidant). Results are summarized in Figure 5.

Singlet oxygen was produced by UVA irradiation of a sensitizer (methylene blue, 10<sup>-5</sup> M). Histidine (10<sup>-2</sup> M) was used as a substrate, which reacts with singlet oxygen to form trans-annular peroxide. This reaction product, in turn, bleaches N,N-dimethyl-p-nitrosoaniline (RNO, 5x10<sup>-5</sup> M in a pH 7.0 phosphate buffer). The bleached form of RNO was measured spectrophotometrically at 440 nm. The singlet oxygen scavenging efficiency of an antioxidant will therefore reduce the amount of free singlet oxygen and thus prevent the bleaching of RNO.

The test was performed under experimental conditions to insure less than 15% of RNO bleaching. This is needed to limit secondary product accumulation, which could contribute to RNO bleaching. The present product concentrations and irradiation time (20 min with a total energy of 12 J/m<sup>2</sup>) were chosen to fulfill this condition.<sup>31,32</sup>

## Chelation of Iron and Copper

Recognizing the importance of available coordination sites in transition metal catalysis,<sup>33</sup> and the crucial role iron plays in initiating oxidative stress to skin, we envisioned that an antioxidant can be a true photoprotective agent provided it chelates all the coordination sites in iron and copper.<sup>34</sup> This is particularly critical because the formation of hydroxyl radicals from superoxide anion or hydrogen peroxide and iron requires only one empty coordination site or a site occupied by a readily dissociable ligand such as water. Water may be completely displaced by stronger ligands like the azide (N<sub>3</sub><sup>-</sup>) anion. We have applied this principle to determine the presence of free coordination site(s) in the Fe<sup>3+</sup>-antioxidant complexes by the UV spectrophotometric method.<sup>34</sup>

Amongst all the Fe<sup>3+</sup>-chelates tested, only the complex of TBPE extract and iron (or copper) showed the absence of any water coordination (that is, the complex is fully and firmly saturated and there is no room for any pro-oxidant activity via the oxo-ferryl or oxo-cupryl radical formation). All other chelators showed disparate coordination site(s), thereby making room for opening oxo-ferryl or oxo-cupryl

<sup>c</sup> Trolox C (Chemical name: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Sigma-Aldrich, Milwaukee, WI

**Table 1. Ultraviolet spectral data of Fe<sup>3+</sup>- and Cu<sup>2+</sup>-chelators. No shift is seen with the TBPE extract.**

Chelator	Absorption Maxima of Complex ( $\lambda_{max}$ in nm)			
	With Fe <sup>3+</sup>	N <sub>3</sub> <sup>-</sup> -Induced Shift	With Cu <sup>2+</sup>	N <sub>3</sub> <sup>-</sup> -Induced Shift
EDTA	241, 283	241, 283, <u>410</u>	240, 278	241, 279, <u>354</u>
TBPE extract	241, 294, 353, 377	241, 294, 353, 377	240, 272, 313	240, 272, 313
Pine Antioxidant	241, 294, 353, 384	241, 294, 353, <u>400, 440</u>	239, 279, 302, 331	239, 280, <u>307, 430</u>
Vitamin C	238, 262	241, 266, <u>295</u>	239, 263	239, 263, <u>284, 364</u>
Grape Antioxidant	247, 295, 353, 396	247, 295, 353, <u>415, 430</u>	240, 277, 328	240, 277, 328, <u>359</u>
Green Tea Antioxidant	240, 272, 324, 390	240, <u>277</u> , 325, 390	241, 276, 327, 403	240, 277, <u>336, 404</u>
Trolox C	240, 284	240, <u>273</u> , 284, <u>360</u>	241, 288	241, <u>261, 352, 440</u>
Gallic Acid	247, 295, 337	247, 295, <u>353, 412</u>	240, 258, 321	240, 258, <u>331, 463</u>

\*The peak positions are obtained from differential spectroscopic scans of 1.0 mM Fe<sup>3+</sup> or Cu<sup>2+</sup> and 5 mM chelator, 1.0M NaN<sub>3</sub>, 50 mM phosphate buffer, pH 7.4, versus the same solution without sodium azide.

radical formation, manifesting pro-oxidant effect, particularly at low concentrations.

Table 1 shows that all complexes except TBPE extract contain at least one coordinated water molecule as shown by the presence of spectral shift(s) induced by sodium azide; only TBPE extract shows no spectral shift induced by sodium azide. The maximum wavelength, the extinction coefficient of the complex, and the association constant between N<sub>3</sub><sup>-</sup> and Fe<sup>3+</sup>-antioxidant/chelator complex are highly variable and depend on the type of bonding between Fe<sup>3+</sup> and chelator, the stereochemistry of the complex, and the number of coordination positions. A complete interpretation of these spectral data, however, is beyond the scope of this article.

### MMP Inhibitory Activity

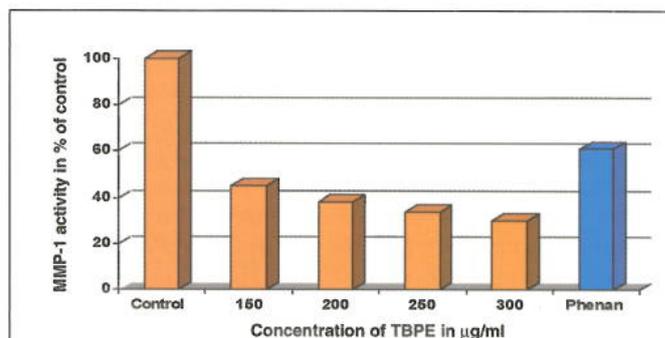
As described earlier, elevated levels of MMP-1, MMP-2 and MMP-3 in skin connective tissue under low-dose UV-irradiation are responsible for the breakdown of various connective tissue components. Although MMP-1 cleaves collagen type I, MMP-2 is able to degrade elastin as well as basement membrane compounds including collagen type IV and type VII. MMP-3 reveals the broadest substrate range for proteins such as type IV, proteoglycans, fibronectin and laminin.

We have investigated the enzyme inhibitory activity of TBPE extract against two MMP-1 and MMP-3. Also, we investigated the inhibitory effect of MMP-1 expression using TBPE extract. Results with protocols are given next.

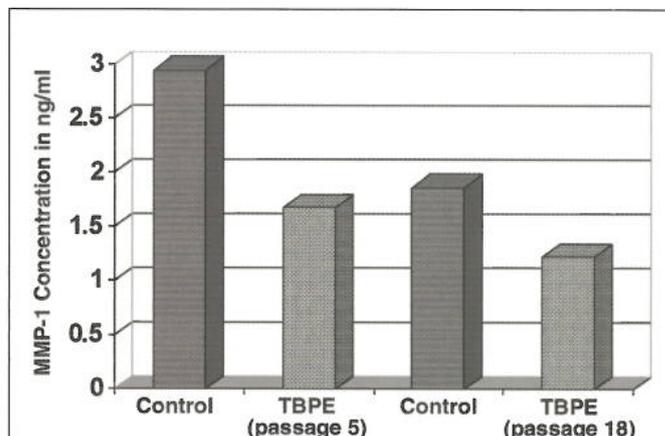
**Collagenase (MMP-1) inhibitory activity:** A dose-dependent inhibition of gelatinase/collagenase activity by approximately 55-70% was observed with the TBPE extract at 150-300 µg/ml. Quantification of gelatinase/collagenase inhibitory activity of the extract was determined by using a gelatinase/collagenase kit<sup>d</sup> and measuring the substrate fluorescence emission at 515 nm. 1,10-Phenanthroline was used as a positive control and collagenase without inhibitor was used as a negative control. Results of this study are summarized in Figure 6.

**Collagenase expression inhibition:** An inhibition of about 40% in collagenase expression was observed using only 50 µg/ml of TBPE extract. Human skin fibroblast cell lines were used for this study. Quantification of collagenase expression was done by using a nonisotopic immunoassay<sup>e</sup> for the in vitro quantification of human matrix metalloprotease (MMP-1, interstitial collagenase).

Experiments were done using human skin fibroblast cells of fifth and eighteenth passages. Data shows a comparable effect of the TBPE extract on MMP-1 (collagenase) expression



**Figure 6. Collagenase (MMP-1) inhibitory activity of TBPE extract. (Phenan = 1,10-Phenanthroline)**



**Figure 7. Inhibitory activity of TBPE extract on MMP-1 expression**

after 48 hrs of incubation in two different experiments. Results are presented in the Figure 7.

**Stromelysin 1 (MMP-3) inhibitory activity:** An inhibition of stromelysin 1 activity by more than 50% was observed with the TBPE extract at 100 µg/ml.

Quantification of MMP-3 inhibitory activity of TBPE extract

<sup>d</sup> E-12055 EnzChek gelatinase/collagenase kit, Molecular Probes, Inc. EnzChek is a registered trade name of Molecular Probes, Inc.

<sup>e</sup> MMP-1 ELISA (Catalog # QIA55) from Oncogene Research Products, Cambridge, MA

was determined by using a stromelysin activity assay kit<sup>f</sup>. The principle of the assay is based upon fluorescence measurement of substrate fragments released upon cleavage of a substrate by MMP-3. Fluorescence intensity of the resulting product is measured and correlated with MMP-3 activity. Results of this study are summarized in the Figure 8.

## Clinical Study

**Background on UV-induced erythema:** Erythema, the most familiar manifestation of UV radiation exposure, occurs in a biphasic manner. UVA and certain visible light mediate the early part of this reaction, known as immediate pigment darkening (IPD), which lasts for about one-half hour. Delayed erythema, a function primarily of UVB dosages, begins 2-8 h after exposure and reaches a maximum after 24-36 h, with erythema, pruritus, and pain in the sun-exposed areas.<sup>35</sup>

Microscopically, changes are detectable as early as 30 min after UV radiation exposure. Epidermal changes include intracellular edema, vacuolization and swelling of melanocytes, and the development of characteristic sunburn cells. In the dermis, UV radiation initially leads to interstitial edema and endothelial cell swelling. Later, there is perivascular edema, degranulation, and loss of mast cells, decrease in number of Langerhans cells, neutrophil infiltration, and erythrocyte extravasation.<sup>35</sup>

**The protocols:** We used reduction in UV-induced erythema as a criterion for photoprotection (Protocol A) and reversal (Protocol B) of photo-damaged skin by the TBPE extract. Test sites were areas 4x2.5 cm on the backs of human subjects. Test substances were creams with 0.2% and 0.5% TBPE extract, 0.5% magnesium ascorbyl phosphate (MAP) or 0.5% vitamin E. The creams were applied once daily at a dose of 2 mg/cm<sup>2</sup>.

Results were represented by using the individual typology angle ITA° (COLIPA SPF test method) obtained by chromometric measurement. ΔE ITA° (difference in skin lightening) was calculated by subtracting the ITA° at the treated irradiated site from the ITA° at the untreated irradiated site. ITA°, the ITA degree, is calculated using the formula

$$ITA^\circ = [\text{Arc Tangent } (L^* - 50)/b^*] \cdot 180 / 3.1416$$

where L\* value is the Lightness value, a\* is the color value in the red-green axis, and b\* is the color value in the blue-yellow axis.

For Protocol A (Prevention), we applied product for 8 days on 11 humans and then on day 9 induced pigmentation by UV light. We compared the untreated irradiated control site versus the product-treated sites on day 10.

For Protocol B (Reversal), we induced pigmentation by UV light on 10 humans and then immediately applied the product and continued product application once a day for 10 days. We compared the untreated irradiated control site versus the product-treated sites every day and found a statistically significant difference in ITA° on day 5.

**Results:** The results are shown in Figure 9. For Protocol A, 0.2% TBPE extract and 0.5% vitamin E showed statistically significant ( $p < 0.05$ ) reduction in erythema. For Protocol B, only 0.2% TBPE extract showed statistically significant reduction in erythema.

## Formulation Guidelines

The TBPE extract can be used in formulations ranging from 0.1% to 1.0% (w/w) level. Typical use levels are 0.1-0.2% for antioxidant formulas and 0.3-1.0% for age-defying applica-

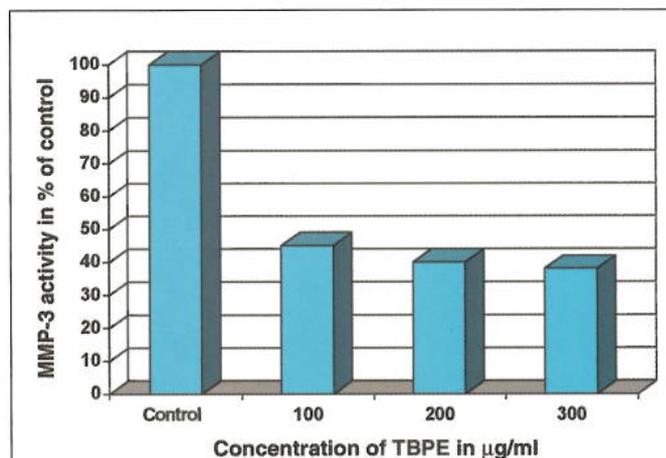


Figure 8. Stromelysin 1 (MMP-3) inhibitory activity of TBPE extract

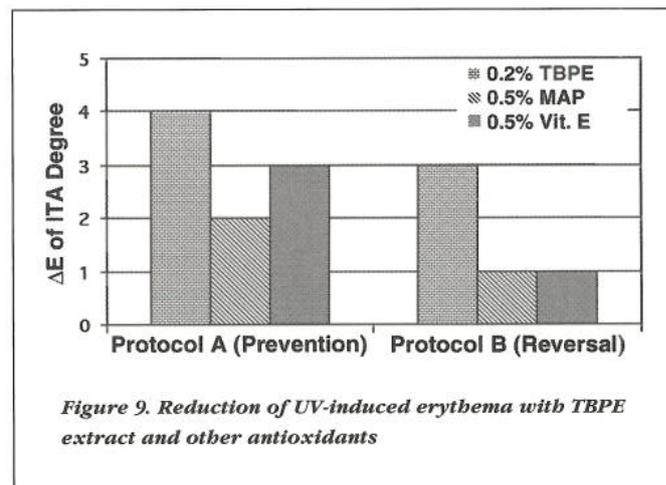


Figure 9. Reduction of UV-induced erythema with TBPE extract and other antioxidants

tions. Acceptable formulation aesthetics can be obtained with use levels of up to 0.5%.

Use of light fragrance may be desired with higher levels of the TBPE extract (>0.5%, w/w). Nonionic or anionic emulsifiers can be used for making stable emulsions. The pH of the formulations must be acidic (preferably, below 5.50) to maintain its antioxidant activity and stability. Addition of a skin penetration enhancer, such as lecithin, may improve the extract's efficacy.

The TBPE extract as a suspension in water can be added to the formulation with moderate agitation at about 40°C. Prolonged heating or exposure to sunlight must be avoided because it causes darkening of formulated products.

## Conclusion

Photoaging of skin is a complex biological process affecting various layers of the skin with major changes seen in the connective tissue of the dermis. The natural shift toward a more pro-oxidant state in intrinsically aged skin can be significantly enhanced by UV-irradiation.

A *Phyllanthus emblica* extract composed primarily of low molecular weight tannins has been shown to reduce UV-induced erythema and has excellent free-radical quenching ability, chelating ability to iron and copper (no pro-oxidative activity) and inhibitory activity against MMP-1 and MMP-3. Possibly, this extract modulates UV/ROS-initiated signal transduction pathways of matrix metalloprotease induction, thereby protecting the extracellular matrix proteins from degradation.

<sup>f</sup> Chemicon MMP-3 / Stromelysin Activity Assay Kit (ECM 481), Chemicon International, Temecula, CA

A properly constituted *Phyllanthus emblica* extract, such as the one described here, may provide a great value as a stand-alone photoprotective agent or in combination with sunscreens for skin care products of the future. Thus, if skin (or sun) care products containing this extract are topically applied before UV-irradiation, at least partial prevention from skin photoaging can be provided.

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## **Emblica Cascading Antioxidant: A Novel Natural Skin Care Ingredient**

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# Emblica Cascading Antioxidant: A Novel Natural Skin Care Ingredient

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Rona/EM Industries, Inc., Hawthorne, N.Y., USA

## Key Words

Antioxidant · Pro-oxidant · Transition metals · Oxidative stress · Skin care · Natural product · Hydrolyzable tannins · *Phyllanthus emblica*

## Abstract

A standardized extract of *Phyllanthus emblica* (trade named Emblica) was found to have a long-lasting and broad-spectrum antioxidant activity. The product has no pro-oxidation activity induced by iron and/or copper because of its iron and copper chelating ability. Emblica helps protect the skin from the damaging effects of free radicals, non-radicals and transition metal-induced oxidative stress. Emblica is suitable for use in anti-aging, sunscreen and general purpose skin care products.

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Emblica (a trade name product of Merck KGaA, Darmstadt, Germany and EM Industries, Inc., USA, an affiliate of Merck KGaA) cascading antioxidant is a well-defined mate-

rial, isolated from the fruits of *Phyllanthus emblica* (syn. *Emblica officinalis* Gaertn). This plant ranges in status from insignificant in the Western world to highly prized in tropical Asia. The tree is graceful ornamental, normally reaching a height of 60 ft (18 m) and in rare instances of 100 ft (30 m). *P. emblica* is one of the important Ayurvedic ('science of life') herbs in India, and has been used for over thousands of years for a wide variety of human ailments [Chopra et al., 1956]. Our study on the antioxidant constituents of *P. emblica* is centered around its skin care benefits, as there has been no systematic work done so far on this product. Product attributes of Emblica are summarized below:

## Product Attributes

- A safe and effective natural antioxidant
- Well-defined material
- Pro-oxidation-free antioxidant
- Dual functionality: chelation and antioxidant, two functions are separated
- Cascading effect provides long-lasting activity

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- Statistically significant reduction in UV-induced pigmentation (results not included)
- Protection of skin fibroblast cells against oxidative stress (in vitro)
- Excellent safety profile – edible fruit.

### Product Standardization

The fruits are selected, harvested and processed according to strict criteria to ensure a consistently high-quality product. The quality criteria are based on extensive research on chemotypes, geographical location of the plants, time of harvesting and on the extraction process to obtain consistently good-quality material. Emblica cascading antioxidant is extracted from premium quality fruits using a water-based process [US Patent No. 6,124,268]. Emblica cascading antioxidant is distinctly different from other commercially available extracts of *P. emblica* fruits, as it is defined to the extent of well over 50% in terms of its key chemical components. None of the extracts of *P. emblica* in the market compares to Emblica cascading antioxidant in composition and consistency of composition, and color.

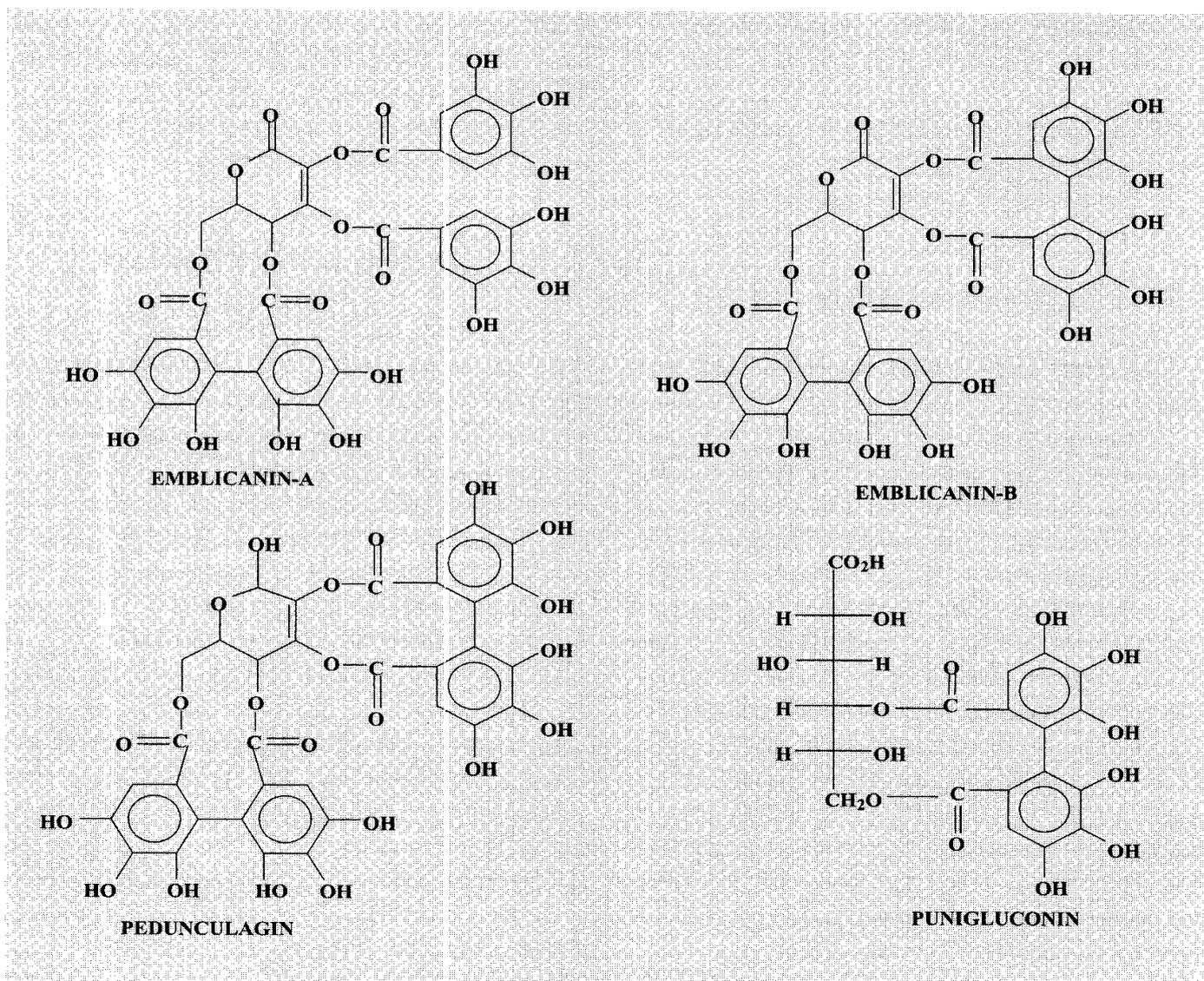
The low-molecular-weight hydrolyzable tannins (<1,000), namely Emblicanin A and Emblicanin B, along with Pedunculagin and Punigluconin are the key ingredients in Emblica cascading antioxidant. The literature continues to claim the presence of stable vitamin C in *P. emblica* as the key ingredient, which has been proven to be erroneous [Ghosal et al., 1996]. In nature, Emblicanin A and Emblicanin B have only been found in *P. emblica* plants [Ghosal et al., 1996]. The structures of the monomeric compounds are given in figure 1.

Emblica cascading antioxidant has been standardized [Monograph, 2001] by using high-performance thin-layer chromatography

with silica gel TLC plate, E. Merck, and a solvent system consisting of ethyl acetate/formic acid/acetic acid/water. Alternately, the product can be standardized by using high-performance liquid chromatography.

### Pro-oxidation-Free Antioxidant

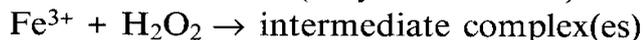
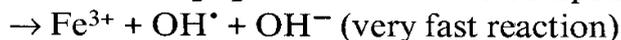
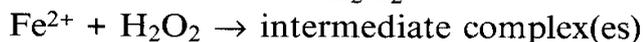
To control oxidative processes, i.e. to reduce, if not prevent, their harmful effects on the skin, diverse antioxidants can be used to protect the skin from photodamage. When a general use of antioxidants is advocated, it is often disregarded that these compounds not only function as antioxidants, but (intrinsically) have a pro-oxidant action [Bast et al., 1991], especially in the presence of transition metals like iron and copper. Release of iron from the iron-storage protein ferritin under UV light has been ascribed to be the main source of oxidative stress [Brenneisen et al., 1998; Pourzand et al., 1999]. The consequent release of potentially harmful free iron within the cells will clearly exacerbate the damaging effects of photoperoxidation and is likely to be of central importance to both reversible and degenerative damage to the skin after exposure to UV light. It has been shown that the iron content of human epidermis is three-fold greater in sun-exposed areas than in nonexposed body sites [Bissett and McBride, 1992]. Iron exerts its toxicity through a series of reactions with reactive oxygen species called the Fenton reaction, generating the highly toxic hydroxyl radical with subsequent damage to biomolecules [Halliwell and Gutteridge, 1998]. Emblica cascading antioxidant is completely free of pro-oxidation activity induced by transition metals, whereas well-known antioxidants like vitamin C, vitamin E, proanthocyanidins (from pine and grape), superoxide dismutase and glutathione do have prooxidative activity [Bast et al., 1991]. Chemis-



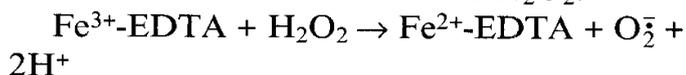
**Fig. 1.** Structures of key ingredients in Emblica cascading antioxidant.

try involved in transition metal-induced pro-oxidation is summarized below:

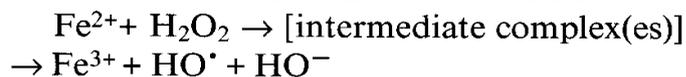
Presence of iron and  $H_2O_2$ :



Presence of  $Fe^{3+}$  chelates and  $H_2O_2$ :



Presence of iron (or copper),  $H_2O_2$  and an antioxidant:



Sunlight also generates superoxide on the skin and increases the presence of superoxide dismutase, which converts the superoxide to hydrogen peroxide.  $Fe^{2+}$  reacts with the hydrogen peroxide to generate the destructive

**Table 1.** UV spectral data of Fe<sup>3+</sup> chelators

Chelator/antioxidant	Absorption maxima of complex, $\lambda_{\max}$ nm	
	with Fe <sup>3+</sup>	N <sub>3</sub> induced shift
EDTA	241, 283	241, 283, <u>410</u>
<b>Emblica</b>	<b>241, 294, 353, 377</b>	<b>241, 294, 353, 377/no shift</b>
Pine antioxidant	241, 294, 353, 384	241, 294, 353, <u>400, 440</u>
Vitamin C	238, 262	241, 266, <u>295</u>
Grape antioxidant	247, 295, 353, 396	247, 295, 353, <u>415, 430</u>
Green tea antioxidant	240, 272, 324, 390	240, <u>277</u> , 325, 390
Gallic acid	247, 295, 337	247, 295, <u>353, 412</u>

The peak positions are obtained from differential spectroscopic scans of 1.0 mM Fe<sup>3+</sup> and 5 mM chelator, 1.0 M NaN<sub>3</sub>, 50 mM phosphate buffer, pH 7.4, versus the same solution without sodium azide. Underlining of figures indicates new peak(s) or peak(s) have been shifted.

hydroxyl radical and Fe<sup>3+</sup>, which allows the chain reaction to continue. In the presence of antioxidants/pro-oxidants like vitamin C, vitamin E or other polyphenolics, one has a perfect oxidative chemistry ready to take place on the skin.

Emblica cascading antioxidant is an excellent chelator for Fe<sup>3+</sup> and Cu<sup>2+</sup>, thereby eliminating the generation of the hydroxyl radical and its detrimental effects on the skin. As an antioxidant, it quenches free radicals that happen to form on the skin. Iron-catalyzed formation of hydroxyl radical from superoxide anion radical and hydrogen peroxide requires the availability of at least one iron coordination site that is either empty or occupied by a readily dissociable ligand such as water. This coordination with water may be completely displaced by stronger ligands like azide (N<sub>3</sub><sup>-</sup>) anion. We have applied this principle and determined if any coordination site is free in the Fe<sup>3+</sup>-antioxidant complex by the UV spectrophotometric method [Graf et al., 1984; Martell et al., 1957]. The results are shown in table 1.

### Cascading Effects

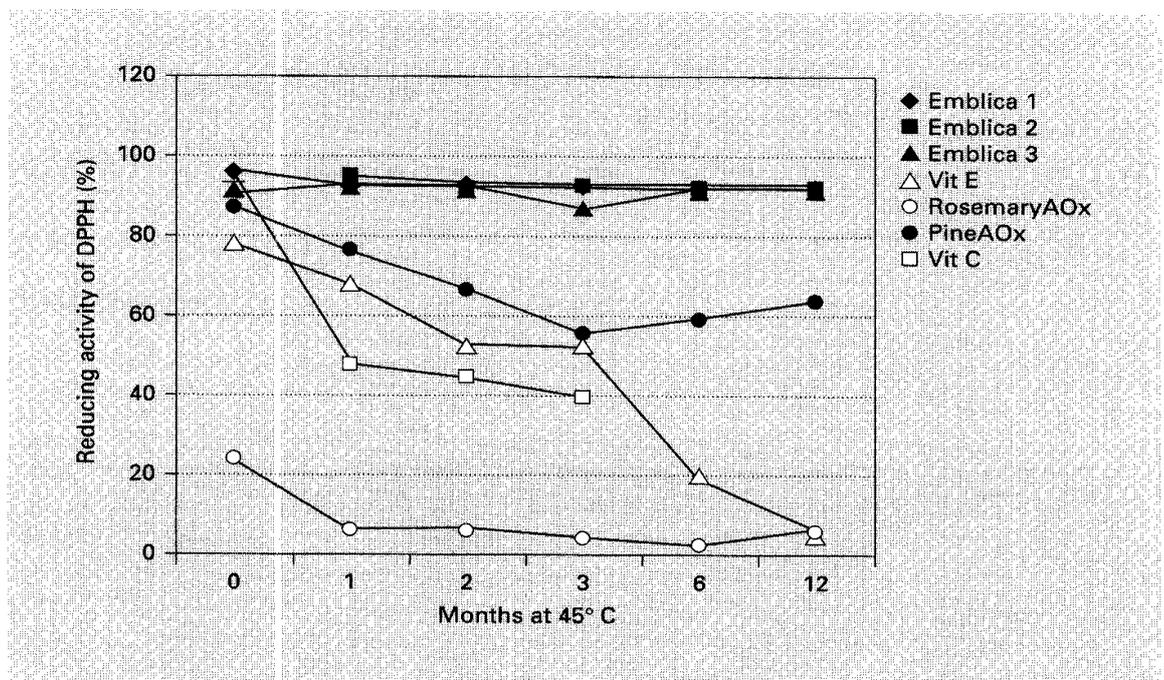
While most antioxidants go from an active to an inactive form, Emblica cascading antioxidant utilizes a multilevel cascade of antioxidant compounds (shown below), resulting in a long-lasting and stable antioxidant activity. This cascading effect was proven by tests of inhibitory activity of Emblica constituents and their metabolites on lipid peroxidation [Halliwell and Gutteridge, 1998; Ohkawa et al., 1979].

Emblicanin A → Emblicanin B → Emblicanin oligomers.

### Stability in Aqueous and Emulsion Systems

The diphenylpicrylhydrazide (DPPH) method [Kato et al., 1988] was used to determine the long-lasting antioxidant activity of Emblica cascading antioxidant. The result is summarized in figure 2.

The DPPH method is a simple colorimetric assay of determining antioxidant activity based on the decrease in absorbance at



**Fig. 2.** Comparative antioxidant activity of Emblica cascading antioxidant and other natural antioxidants. All antioxidant activities were derived from optical density measurements at 517 nm in ethanol-water with concentrations as follows: DPPH at 100  $\mu\text{M}/\text{ml}$ ; all antioxidants at 31  $\mu\text{g}/\text{ml}$  except rosemary antioxidant at 61  $\mu\text{g}/\text{ml}$ . Vit = Vitamin; RosemaryAOx = rosemary antioxidant; PineAOx = pine antioxidant.

517 nm of the DPPH radical (deep purple) after the addition of an antioxidant compound in an aqueous ethanolic solution. Emblica cascading antioxidant retains antioxidant activity to the fullest extent after 12 months at 45°C, while others failed. Additionally, a minimal batch-to-batch variation in antioxidant activity of Emblica cascading antioxidant is seen in this test.

xanthine oxidase system [Richard et al., 1992] to generate superoxide to determine protection of human dermal fibroblast cells using Emblica cascading antioxidant. Cell viability under oxidative stress was determined by measuring optical density at 570 nm. The results are summarized in table 2. Emblica provides about 87% protection of human dermal fibroblast cells under oxidative stress.

### Protection of Skin Fibroblast Cells under Oxidative Stress (in vitro)

The direct biological damage that can be caused by superoxide is highly selective and often involves its reaction with other radicals. Thus, superoxide can reduce  $\text{Fe}^{3+}$ , but also oxidize  $\text{Fe}^{2+}$ . We have used the hypoxanthine-

### Safety Profile

#### Acute Oral Toxicity Study in Rats – Limit Test

An acute oral toxicity test was conducted with rats to determine the potential for Emblica to produce toxicity from a single dose via the oral route. Under the conditions of this

**Table 2.** Protective effect of Emblica cascading antioxidant on human skin fibroblast cells under oxidative stress

	Control	HX-OX	Emblica 1	Emblica 2
Optical density at 570 nm	0.755	0.22	0.68	0.63
Protection of cells against oxidative stress	–	–	90%	83%

HX-OX = Hypoxanthine-xanthine oxidase.

study, the single-dose acute oral LD<sub>50</sub> is >5,000 mg/kg of the body weight in male and female rats.

#### *Primary Eye Irritation Study in Rabbits*

A primary eye irritation test was conducted with rabbits to determine the potential for Emblica to produce irritation from a single instillation via the ocular route. Under the conditions of this study, the test substance is classified as minimally irritating to both the unrinsed and rinsed eyes.

#### *Evaluation of Phototoxicity Potential by UVA Irradiation on Human Subjects*

A phototoxicity test was conducted with 20 human subjects to determine the potential for Emblica to produce phototoxicity from a single dermal application. Under the conditions of this study, the test substance is classified as nonphototoxic when tested on human subjects at 2% dilution in distilled water.

#### *Repeat Insult Patch Test on Human Subjects/Skin Irritation and Skin Sensitization Evaluation*

A repeat insult patch test was conducted with 100 human subjects to determine the potential for Emblica to produce primary irritation and primary sensitization. Under the conditions of the study, the test substance is considered as a nonprimary irritant and a nonprimary sensitizer to the human skin.

#### *Salmonella Mutagenicity Test*

A salmonella mutagenicity test was conducted to determine the potential for Emblica at 50 and 100 µg/plate to produce mutagenicity. Under the conditions of the study, the test substance is considered as a nonmutagenic material.

#### **Conclusion**

Emblica cascading antioxidant has an excellent toxicity profile and has no adverse biological effects on humans. Thus, Emblica cascading antioxidant is a safe, suitable and effective product for use in antiaging, sunscreen and general-purpose skin care and skin protection products as well as in color cosmetics. Emblica cascading antioxidant, having no pro-oxidation property, may provide a greater value than the existing antioxidants as a stand-alone or in combination with sunscreens for skin care products of the future.

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## Turn on Bright Skin – Formulation Options

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## Turn on Bright Skin – Formulation Options

### ■ Introduction

Nowadays whitening ingredients are currently available in a variety of facial skin care products from acne treatments and face masks to toners. This property continues to be the strongest in facial

moisturizers accounting for over half of global whitening sales. Dark spots, uneven skin tone and sun damage are skin imperfections which continue to rule skin concerns of Asian consumers. But this demand is not specific to Asian countries. A »perfect skin complexion« representing

the symbol of a younger appearance is also valid everywhere in the world. Psychological studies conclude that a freckled and mottled skin adds up to twenty years in age perception independent of the age of the individual (1–3).

An old proverb says »You never get a second chance to make a first impression«. This is especially true in terms of one's valuation by other people based on facial appearance during the first contact. A fresh and healthy skin complexion is a door opener in private as well as in business life. Skin appears most attractive when it is luminous white, while yellowness is perceived as a sign of ageing. Therefore it is one of the main motivations of cosmetic raw material manufacturers to provide new ingredients designed to ensure a flawless and radiant skin complexion, to correct hyperpigmented skin and to achieve a homogenous and bright skin appearance.

A variety of compounds and extracts able to brighten human skin have been known for many years (3–6). Especially hydroquinone was extensively used in the past. Nowadays the safety aspect of skin brightening ingredients plays a quite important role. When applying cosmetic products on a daily basis the formulator should ensure in the goal of achieving a long lasting brightening impact that no side reactions e.g. like itching or sensitization of skin should occur. In Table 1 a short summary of drawbacks of established skin brightening agents is shown. Although several ingredients clearly pose a risk for skin application, they are still widely used (2).

A top 10 list of the most frequently used skin brightening ingredients revealed that kojic acid remained the ingredient of

### Abstract

**G**lobally the continuous interest in skin care products using active ingredients to improve skin complexion remains high. Especially in Asia Pacific an ongoing »love affair« with porcelain-like skin is a key influence in skin care innovation. The desire for lighter skin is constantly increasing while skin protection remains equally important and is an aspect one needs to keep in mind. (1)

Flawless and bright skin complexion as an expression of youth and healthiness is highly desired by women of all ages in many modern societies and cultures. For this reason the cosmetic industry is constantly searching for new effective, safe and easy to formulate skin brightening/lightening/whitening agents. This paper will introduce the new brightening ingredient Methoxyphenyl t-Butylphenyl Propanediol and highlights its efficacy based on a clinical study. This innovative ingredient underlines its unique position among skin brighteners by shifting human skin tone towards a fairer complexion with a reduction of yellowness as observed *in vivo*. It provides an option to achieve a radiant porcelain-like skin complexion and to simultaneously improve skin freshness and healthy appearance. Beyond discussion of the product benefits of this innovative compound, formulation guidance is provided to further achieve an enhanced radiant skin complexion. The result will assist the formulator to create modern and appealing products with skin brightening ingredient combinations resulting in dramatic impact on skin complexion.

choice while mulberry extract accounted for an attractive natural option providing powerful brightening properties. Next to alpha- and beta-Arbutin – holding place 3 and 4 – one can also identify Vitamin A and Vitamin B3 (Nicotinamide) in the top 10 list. Vitamin C derivatives ranked lower but are well-established alternatives to brighten skin (3).

Taking a closer look at market products one can identify sophisticated combinations of skin brightening actives providing further skin protecting properties as actual demands are requesting additional properties like antioxidant efficacy, moisture supporting options or pore size reducing effect. Human skin brightening process is usually focused on the reduction of melani-

$ITA^\circ > 55^\circ$	»Very Light«	skin type I
$55^\circ \geq ITA^\circ > 41^\circ$	»Light«	skin type II
$41^\circ \geq ITA^\circ > 28^\circ$	»Intermediate«	skin type III
$28^\circ \geq ITA^\circ > 10^\circ$	»Tan/Matt«	skin type IV
$10^\circ \geq ITA^\circ > -30^\circ$	»Brown«	skin type V
$ITA^\circ < -30^\circ$	»Dark brown«	skin type VI

Fig. 2 Different skin types based on  $ITA^\circ$  values.

nin content in the epidermis. Ingredients performing that way help to achieve an overall lightening of the skin tone but do not enhance the radiance or luminosity of the skin. To generate a fresh and young looking complexion it is necessary

to increase the luminous power of the skin itself, by influencing all parameters that determine skin color.

Skin color is described by 3 basic parameters defined by the CIE System  $L^*$ ,  $a^*$ ,  $b^*$  values (The Wikipedia Link provided a detailed description of this system):  $L^*$  represents the black to white color change component indicating how light or dark a color is,  $a^*$  represents the red to green color component and  $b^*$  the yellow to blue component. The hue represents the color combining  $a^*$  and  $b^*$  values for example: red, purple, blue, etc. The chroma (saturation) represents strength or dominance of the hue.

With these values the color of any system can be described and compared. The effect of most known skin brightening ingredients is based on their capabilities to shift the black/white scale determining parameter  $L^*$  to higher numbers – meaning a less dark skin. Though that is helpful for very dark skin complexions or hyperpigmented areas it neither provides enhanced luminosity nor is useful for people with a quite fair but yellowish skin appearance. Especially people with a very strong yellow color component in their skin complexion would appreciate a shift towards a less saturated yellowish skin tone in order to achieve not only a fair but especially a luminous skin appearance. This highlights the high importance of the  $b^*$ -parameter in the overall performance of a skin brightening ingredient. Chardon *et al.* defined the different skin types based on the individual typology angles ( $ITA^\circ$ ) which were included in the COLIPA regulations (7-10). These  $ITA^\circ$  values were calculated using the  $L^*$  and  $b^*$  parameters (Fig. 1).

Although the importance of the skin color parameter  $b^*$  has been known for more than two decades not much attention has been specifically paid to this factor in the

Compound	Obstacle	Comment
Hydroquinone	Potential skin sensitizer, toxic/suspected to be carcinogenic to kidneys/Ochronosis (bluish-black discoloration of certain tissues)	Restricted use or even ban in many key markets. FDA request for additional data
Kojic acid	Potential skin sensitizer, toxic to thyroid	Increased skin sensitization potential in several studies reported
Arbutin	Discussed as hydroquinone precursor	Products containing $\beta$ -arbutin regarded as »unsafe« by SCCP (2008); $\alpha$ -arbutin also in discussion for adverse effects
Ascorbic acid	Potential low efficacy	Unstable; tends to cause discolorations in the final formulation
$\alpha$ -Hydroxy acids	Potential low efficacy	Causes skin irritation due to low pH

Table 1 Drawbacks of established skin brightening agents.

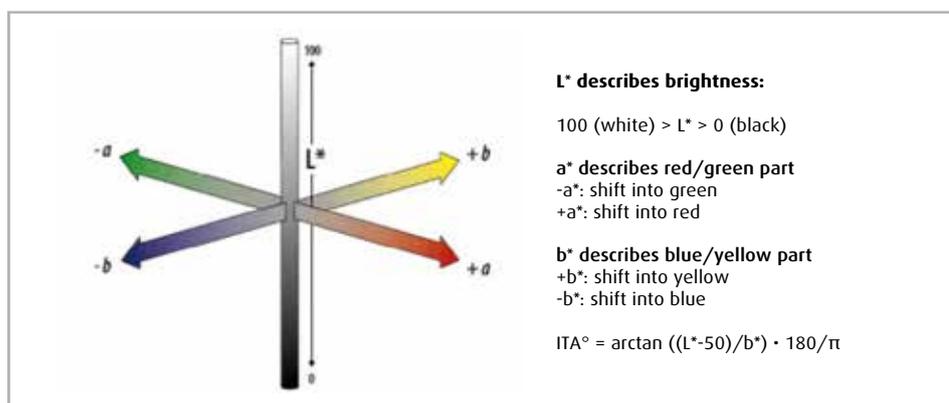


Fig. 1 Calculation of individual typology angle ( $ITA^\circ$ ) based on skin color parameters  $L^*$  and  $b^*$ . Based on the  $ITA^\circ$  values 6 skin types were defined (Fig. 2).

development of new cosmetic ingredients for skin brightening.

Herein we present an innovative cosmetic active that is tailored to brighten human skin not only by slowing down the biogenesis of the natural skin pigments called melanins, but also by reducing yellowness by shifting the skin color to a whiter and more porcelain-like appearance.

The intention of this paper is to demonstrate the effectiveness of this innovative ingredient and to furthermore highlight its versatile use. The variety of new formulation options having this safe and stable ingredient on hand enriches formula-

tor requests for modern skin brightening products. The comparison of this substance with established skin brighteners clearly indicate the attractiveness of this new molecule offering a safe and effective alternative to improve skin complexion.

■ Efficacy Aspects

**In vivo study on pigmentation reduction**

The study was carried out with a panel of 17 volunteers with an average age of 50 years. The initial ITA° values varied from

10° to 45° corresponding to Skin Type II to IV according to the skin type classification (Fig. 2). The study took place in the north of Germany during a period of 3 weeks in late spring. Test and reference emulsions were applied twice daily to test areas defined on the inner forearms of participating individuals. Four test areas were defined for each volunteer. Test areas were either untreated or treated with emulsions containing 2-ascorbyl-glucoside (Formulation A) or Methoxyphenyl t-Butylphenyl Propanediol (BP) at 1.0 % wt (Formulation B) and 0.3 % wt (Formulation C) as listed in Table 2. The read-out parameters in this case were the skin color components L\* and b\* as obtained by chromametric measurements at t=0 and t=3 weeks. ITA° angles were calculated using the previously mentioned equation.

The study focused on 2 topics:

- the reduction of melanin content in skin and
- the observation of the modification of the skin color parameter b\*

The skin color impacting properties of MBP were compared to the one of the benchmark 2-ascorbyl glucoside. It was found that MBP was clearly superior to 2-ascorbyl glucoside to reduce the melanin biosynthesis. The new cosmetic active MBP showed a clear tendency to brighten the skin after 22 days (Fig. 3).

Although brightening of the skin is a strong and important demand by consumers, we think there is another need that has not yet been addressed. Skin may be light in color but may not always appear appealing and fresh. This lack of attraction is caused by changes in the skin color parameters underlying the overall skin tone. This is particularly true for light skin that has a fair but yellowish complexion. Examination of skin color parameter b\* in the presented in vivo study showed that MBP (1 %) showed a better performance than 2-ascorbyl glucoside (2 %) (Fig. 4).

The untreated skin area showed a measurable yellowing of the skin. The study was carried out end of spring when high UV indices started to trigger skin tanning. Benchmark 2-ascorbyl glucoside (2 %) only caused a weak effect in the

PHASE	INGREDIENTS (INCI)	A (%)	B (%)	C (%)
A	METHOXYPHENYL T-BUTYLPHENYL PROPANEDIOL	0.00	1.00	0.30
	ARACHIDYL ALCOHOL, BEHENYL ALCOHOL, ARACHIDYLGLUCOSIDE	1.00	1.00	1.00
	DIETHYLHEXYL CARBONATE	5.00	5.00	5.00
	HEXYL LAURATE	5.00	5.00	5.00
B	AQUA (WATER)	77.60	79.00	79.70
	GLYCERIN	5.00	5.00	5.00
	ASCORBYL GLUCOSIDE	2.00	0.00	0.00
C	LAURETH-7, POLYACRYLAMIDE, C13-14 ISOPARAFFIN	3.00	3.00	3.00
D	AQUA, SODIUM HYDROXIDE	0.40	0.00	0.00
	PHENOXYETHANOL, ETHYLHEXYL GLYCERIN	1.00	1.00	1.00
TOTAL		100.00	100.00	100.00

Table 2 O/W test emulsions used for in vivo studies.

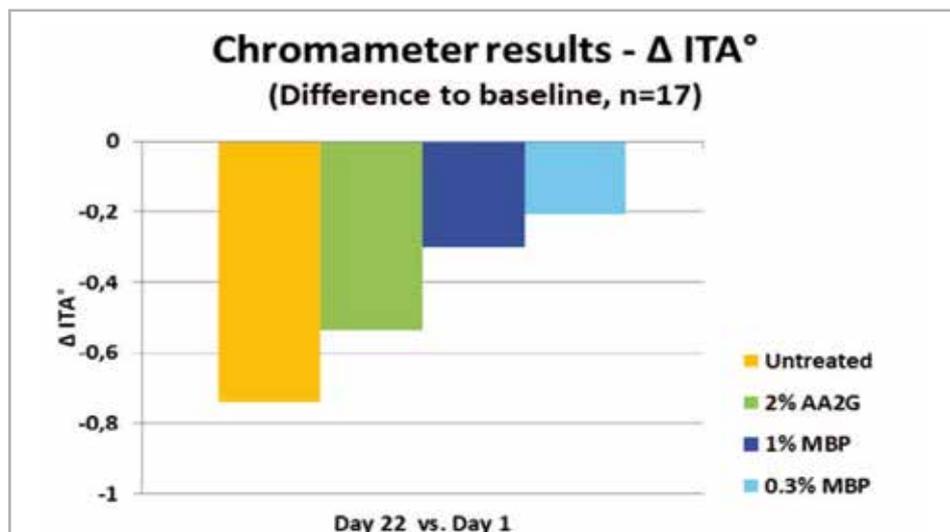


Fig. 3 Prevention of skin pigmentation – Impact on ITA° value by new cosmetic ingredient MBP.

overall inhibition of melanogenesis and in the prevention of skin yellowing. Only the new ingredient MBP was able to cause a change in the  $b^*$  value towards a lower yellow saturation in skin color. It was also clearly shown that the manifestation of shifting the  $b^*$  parameter of skin color towards less yellow took place in a dose-dependent manner as shown in Fig.4 with the delta  $b^*$ -value change.

It was found that the new skin brightening ingredient MBP differentiates itself from known skin color modulating cosmetic actives by its unique ability to shift the skin color from a yellowish complexion to a luminous porcelain-like skin tone. This is linked to an enhancement of freshness and radiance in the overall appearance of skin.

For the time being we can only speculate to explain the rationale behind the yellowness reduction in skin color. The search for an explanation of this mechanism will be the topic of further investigations. One hypothesis is that MBP is affecting the ratio of pheo- and eumelanins in such a way that this shift occurs, having a positive impact on skin complexion.

### ■ Formulation Options

As mentioned above the market demand for efficient and powerful products to improve skin brightening and to make it visible is constantly high. A variety of different actives is available but the compatibility of the suitable ingredients within a formulation remains the limiting factor. The simplest approach is to combine appropriate brightening actives in one formulation to achieve safe and attractive market products. Furthermore combining actives with different mode of action offers the advantage to increase the efficiency of the formulation by targeting different biochemical pathways simultaneously.

To carry out attractive and market-fitting skin brightening products we chose the following ingredients for smart and effective combinations.

- Emblica® – a natural extract out of the Amla fruit
- Nicotinamide (RonaCare® Nicotinamide) – a well-established vitamin B<sub>3</sub>

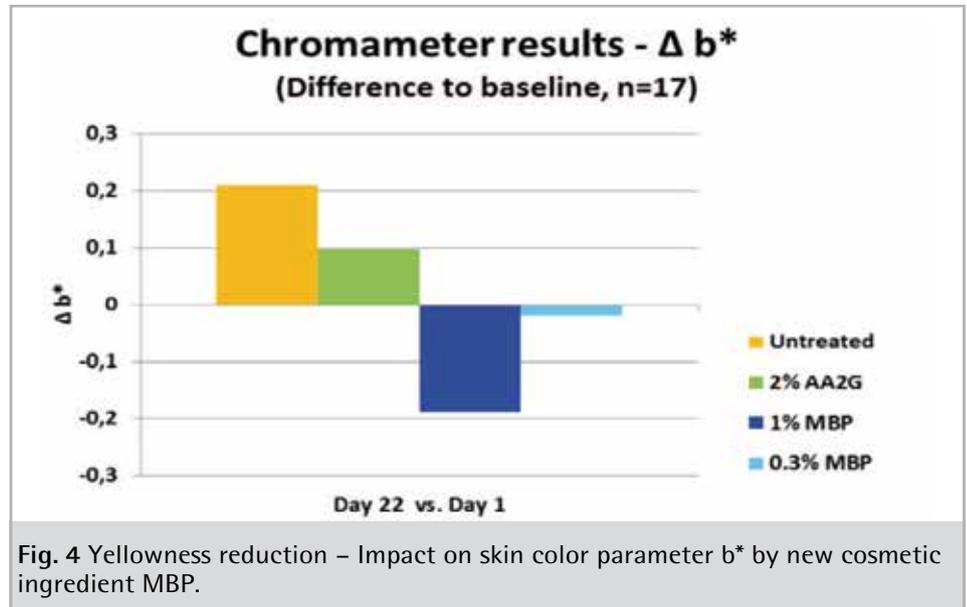


Fig. 4 Yellowness reduction – Impact on skin color parameter  $b^*$  by new cosmetic ingredient MBP.

- Magnesium ascorbyl phosphate (RonaCare® MAP) – as a stable Vitamin C derivative
- Methoxyphenyl t-Butylphenyl Propanediol (RonaCare® Pristine Bright™) – a new safe, stable and effective skin brightening agent

### ■ Formulation Recommendations

#### Formulating with *Emblica Phyllanthus* (Fruit) Extract:

*Emblica Phyllanthus* (Fruit) Extract (Emblica®) is a natural and safe extract gained from the *Phyllanthus emblica* fruits by a water-based process. It offers very good antioxidant and skin brightening properties by acting at several sites in the melanogenesis pathway. Key bio-active ingredients responsible for the inhibition of the pigmentation formation process are its low molecular hydrolysable tannins.

The recommended use level of *Emblica Phyllanthus* (Fruit) Extract for skin brightening effects is 1 to 2%. Non-ionic or anionic emulsifiers can be used to develop stable oil-in-water or water-in-oil emulsions. For the development of gels, electrolyte tolerant gelling agents should be selected.

For incorporation in emulsions, *Emblica Phyllanthus* (Fruit) Extract can be best

added as a pre-dispersion in water with a moderate agitation below 40 °C. To maintain its activity and stability, the pH of the formulation must be acidic, preferably below 5.5. *Emblica Phyllanthus* (Fruit) Extract is an off-white to fawn powder with characteristic odor resulting to off-white to beige oil-in-water emulsions that may darken over time, especially at higher temperature. For this reason, the finished product should be protected from light and heat.

Color improvement of the finished product can be achieved by addition of small amount of sodium metabisulfite ( $\leq 0.1\%$ ) along with ascorbic acid ( $\leq 0.05\%$ ). Alpha hydroxy acids support the efficacy of lightening formulations and reduce a possible color shift of the product as well. Moreover combinations with high covering functional fillers offer options to realize whiter formulations. *Emblica Phyllanthus* (Fruit) Extract is compatible with other skin lightening agents as long as the pH of the formulation is acidic. Therefore such ingredients like magnesium or sodium ascorbyl phosphate that require neutral pH conditions should not be included in the formulation.

As *Emblica Phyllanthus* (Fruit) Extract is a very good chelator of iron and copper ions, contact with those metals should be avoided and manufacturing should be conducted in stainless steel vessels.

**Formulating with Niacinamide:**

Niacinamide (RonaCare® Nicotinamide) is a water-soluble multi-talented ingredient of the vitamin B group (vitamin B<sub>3</sub>). Besides many well-reported beneficial effects on skin conditions, it provides skin brightening properties by inhibiting the transport of the melanin-containing melanosomes from the melanocytes to the keratinocytes of the epidermis. Niacinamide is a white odorless crystalline powder freely soluble in water and ethanol.

It is stable towards oxygen, light, heavy metals and at a pH range from 5.0 to 7.5. More acidic or more alkali environments should be avoided as Niacinamide would be hydrolyzed to nicotinic acid which is far less water-soluble (crystallization could occur) and might result in skin irritation.

Niacinamide can be added to the water phase of emulsions and gels and be heated up to 80 °C. Use levels up to 5 % can be considered.

The high solubility, good compatibility and specific mode of action influencing the biological pathway of pigmentation of Niacinamide make it an ideal ingredient to enrich any kind of water-based skin brightening product.

**Formulating with Magnesium**

**Ascorbyl Phosphate:**

Magnesium Ascorbyl Phosphate (RonaCare® MAP) is obtained *via* an enzymatic process by a regiospecific phosphorylation of vitamin C to yield the magnesium salt of the 2-phosphate ester of L-ascorbic acid. It represents a highly stable derivative of Vitamin C that is used for its antioxidant, anti-aging and skin lightening proper-

ties. Unlike L-ascorbic acid, it is stable when exposed to air or light and does not readily degrade in the presence of water. Therefore it offers a solution for developing formulations with a vitamin C that remains perfectly stable for the long-term. Magnesium Ascorbyl Phosphate is a white to pale yellowish non-odorous powder. It has good solubility in water and can be added either directly to the water phase of a formulation prior to heating or as a pre-dissolved solution in water at the end of the formulation process.

As Magnesium Ascorbyl Phosphate may degrade in the presence of metal ions, chelating agents (e.g. disodium-EDTA, sodium citrate) should be used. For emulsions, nonionic emulsifiers are recommended to avoid any interaction with this high electrolytic ingredient. When formulating gels, electrolyte tolerant gelling agents should

PART	TRADE NAME	INCI NAME	(%)
A	RonaCare® Pristine Bright™ (Merck)	METHOXYPHENYL T-BUTYLPHENYL PROPANEDIOL	1.00
	Eusolex® T-S (Merck)	TITANIUM DIOXIDE (NANO), ALUMINA, STEARIC ACID	8.00
	RonaCare® AP (Merck)	BIS-ETHYLHEXYL HYDROXYDIMETHOXY BENZYL MALONATE	2.00
	KF-6017 (Shin Etsu)	PEG-10 DIMETHICONE	4.00
	Dow Corning 9040 Silicone Elastomer Blend (Dow corning)	CYCLOPENTASILOXANE, DIMETHICONE CROSSPOLYMER	5.00
	Dow Corning FZ-3196 (Dow corning)	CAPRYLYL METHICONE	12.00
	Dermofeel sensolv (Dr. Straetmans)	ISOAMYL LAURATE	3.00
B	RonaCare® Nicotinamide (Merck)	NIACINAMIDE	3.00
	RonaCare® Ectoin (Merck)	ECTOIN	0.50
	Water, demineralized	AQUA	ad 100
	Sodium Chloride (Merck)	SODIUM CHLORIDE	1.00
	1,2-Propanediol (Merck)	PROPYLENE GLYCOL	3.00
	Complexing agent		q.s.
	Preservatives		q.s.
C	RonaFlair® LDP White (Merck)	SODIUM POTASSIUM ALUMINUM SILICATE, CI 77891, SILICA	3.00
	Timiron® SynWhite Satin (Merck)	SYNTHETIC FLUORPHLOGOPITE, CI 77891, TIN OXIDE	0.20
	Colorona® Imperial Topaz (Merck)	MICA, CI 77163, CI 77491, CI 77492, CI 77499	0.20
	SAT-R-33128 (Miyoshi)	CI 77491, DIMETHICONE	0.26
	SA-C335000-10 (Miyoshi)	CI 77499, DIMETHICONE	0.15
	MiBrid Color Powder SA/NAI-Y-10 (Miyoshi)	CI 77492, DIMETHICONE, DISODIUM CAPRYLOYL GLUTAMATE	0.60
	MiBrid Color Powder SA/NAI-TR-10 (Miyoshi)	CI 77891, DIMETHICONE, DISODIUM CAPRYLOYL GLUTAMATE	1.00

Appearance: Chocolate milk tinted lotion

Viscosity (22 °C): 12,000 mPas (Brookfield RVT,DVII+, Helipath spindle C, 10 rpm)

Stability: 3 months at 40 °C, FTC (-5 °C to 40 °C), 5 °C, RT and 1 week at 50 °C

**Formulation 1** Beauty Color Control – W/Si emulsion with Methoxyphenyl t-Butylphenyl Propanediol and Niacinamide.

be selected and polymeric emulsifiers should be pre-neutralized before adding Magnesium Ascorbyl Phosphate. It is also important to adjust the pH of the final product at a neutral value as Magnesium Ascorbyl Phosphate may discolor in formulations with a pH below 6. For this reason, combinations with the above cited *Embllica*<sup>®</sup> are not suitable. The recommended use level of Magnesium Ascorbyl Phosphate for skin brightening purposes is up to 3%.

#### Formulating with Methoxyphenyl t-Butylphenyl Propanediol:

Methoxyphenyl t-Butylphenyl Propanediol (*RonaCare*<sup>®</sup> Pristine Bright<sup>™</sup>) is a highly pure synthetic active which is very easy to handle. It appears as a white odorless powder that allows the development of snowy/pristine white formulations. The particularity of Methoxyphenyl t-Butylphenyl Propanediol is that it is oil-soluble unlike the vast majority of existing skin brighteners. Thanks to its high heat stability and high solubility in cosmetic solvents

(best in polar emollients and liquid UV filters), it can be easily incorporated into the oil phase of oil-in-water or water-in-oil emulsions and gels. Recommended use levels are 0.3% to 2%. Due to the high compatibility of Methoxyphenyl t-Butylphenyl Propanediol with cosmetic raw materials (no incompatibility known so far), numerous combination options with any kind of other active ingredients - including skin brighteners - are possible, thus providing almost unlimited freedom to formulate. Methoxyphenyl t-Butylphenyl Propanediol can indeed be incorporated in any product having an oil phase and can be therefore suitable for decorative cosmetics.

#### ■ Attractive Skin Brightening Formulations

As the global trend to use BB or CC creams for radiance with enriched skin protection is still lasting, we chose a

water-in-silicone CC formulation to demonstrate the compatibility of two skin brightening ingredients Nicotinamide and the new stable and safe Methoxyphenyl t-Butylphenyl Propanediol. The oil soluble skin brightener could be incorporated into the silicone phase/framework/network of the formulation. The dual approach meaning one skin brightener active in the water phase and the other one in the silicone phase results in a smart and simple formulation processing. This formulation is in addition enriched with a powerful antioxidant, an inorganic UV filter and a functional filler to achieve immediate perfection. Selected low tinting interference pigments define a beautiful skin color shade to be used on different skin types (**Formulation 1**).

The combination of Methoxyphenyl t-Butylphenyl Propanediol (*RonaCare*<sup>®</sup> Pristine Bright<sup>™</sup>) with a natural *Embllica Phyllanthus* (Fruit) Extract could be carried out in the **Formulation 2**. By taking

PART	TRADE NAME	INCI NAME	(%)
A	<b>Embllica<sup>®</sup> (Merck)</b>	<b>PHYLLANTHUS EMBLICA EXTRACT</b>	<b>2.00</b>
	Water, demineralized	AQUA	ad 100
	Tris(hydroxymethyl)-aminomethane (Merck)	TROMETHAMINE	0.50
	Complexing agent		q.s.
	Rhodicare S (Rhodia)	XANTHAN GUM	0.50
B	<b>RonaCare<sup>®</sup> Pristine Bright<sup>™</sup> (Merck)</b>	<b>METHOXYPHENYL T-BUTYLPHENYL PROPANEDIOL</b>	<b>1.00</b>
	RonaCare <sup>®</sup> AP (Merck)	BIS-ETHYLHEXYL HYDROXYDIMETHOXY BENZYL MALONATE	1.00
	Cetiol B (BASF)	DIBUTYL ADIPATE	8.00
	Tegosoft TN (Evonik)	C12-15 ALKYL BENZOATE	8.00
	Myritol 331 (BASF)	COCOLYGLYCERIDES	3.00
	Tegosoft liquid (Evonik)	CETEARYL ETHYLHEXANOATE	2.00
	Xiameter <sup>®</sup> PMX-200 (100cs) (Dow Corning)	DIMETHICONE	2.00
	Jojoba Oil (Henry Lamotte)	SIMMONDSIA CHINENSIS	1.00
	Pemulen TR-2 (Lubrizol)	ACRYLATES/C10-30 ALKYL ACRYLATE CROSSPOLYMER	0.20
C	Eusolex <sup>®</sup> UV-Pearls <sup>®</sup> OB-S (Merck)	AQUA, OCTOCRYLENE, SORBITOL, BUTYL METHOXY-DIBENZOYLMETHANE, SILICA, PVP, CHLORPHENESIN, PHENOXYETHANOL, DISODIUM EDTA	12.00
	Preservatives		q.s.
	Fragrance	PARFUM	q.s.

Appearance: Light beige gel. pH (22 °C): 5.3

Viscosity (24 °C): 12,000 mPas (Brookfield DVII+ Pro, Helipath spindle B, 10 rpm)

Stability: 3 months at 40 °C, FTC (-5 °C to 40 °C), 5 °C, RT and 1 week at 50 °C

Store in opaque packaging.

**Formulation 2** Neck & Décolleté Care – O/W gel with Methoxyphenyl t-Butylphenyl Propanediol and natural extract *Phyllanthus Emblica* Extract.

the above mentioned formulation recommendations for the natural extract into account like e.g. incorporating the extract into the water phase combined with an appropriate rheology modifier one ends up with a light beige gel offering natural power to brightening skin plus antioxidant protection. The oil phase is furthermore enriched with skin barrier supporting agents. An encapsulated UV filter system plus a multifunctional synthetic antioxidant complete the overall protection of delicate skin with this potent formulation.

An alternative fresh and modern formulation may be prepared using M. Ascorbyl Phosphate, Nicotinamide and the new skin brightener Methoxyphenyl t-Butylphenyl Propanediol (RonaCare® Pristine Bright™). This combination of 3 different skin brightening actives associated to an anti-aging liposome based unique cyclic peptide (RonaCare® Cyclopeptide-5) and a functional filler provide a smooth and pleasant application and feel on skin

that is easy spreadable. It is a light lotion to be used at all age stages of life. The formulation (Formulation 3) is expected to work *via* different melanogenesis impacting pathways.

■ Summary

Flawless, bright and radiant skin is one of consumer’s strongest demands in today’s cosmetic business. The desire for a homogenous luminous skin complexion is driven by scientific evidence that such an appearance contributes substantially to age perception. Herein it has been proven by a clinical study that the new cosmetic active ingredient Methoxyphenyl t-Butylphenyl Propanediol has a substantial contribution to modulate skin complexion towards radiant and porcelain-like appearance. Skin was brightened and unattractive yellowness was counteracted by inducing a yellowness reduction meaning a shift toward a porcelain skin color as

shown by the change of the color parameter *b\**. The formulation aspect of this stable and safe skin brightener was further evaluated in combination with other established skin brightening ingredients. All formulations represent stable options with high elegance for market use. Further data assessing efficacy on hyperpigmentation are under evaluation and will complete the overall set up of data regarding the new skin brightening active ingredient Methoxyphenyl t-Butylphenyl Propanediol.

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PART	TRADE NAME	INCI NAME	(%)
A	RonaCare® Pristine Bright™ (Merck)	METHOXYPHENYL T-BUTYLPHENYL PROPANEDIOL	1.50
	RonaCare® AP (Merck)	BIS-ETHYLHEXYL HYDROXYDIMETHOXY BENZYL MALONATE	0.50
	Montanov S (Seppic)	COCO-GLUCOSIDE, COCONUT ALCOHOL	3.00
	Montanov 202 (Seppic)	ARACHIDYL ALCOHOL, BEHENYL ALCOHOL, ARACHIDYL GLUCOSIDE	1.00
	Tegosoft DEC (Evonik)	DIETHYLHEXYL CARBONATE	8.00
	Crodamol IPIS (Croda)	ISOPROPYL ISOSTEARATE	7.00
	RonaFlair® Boroneige® SF-3 (Merck)	BORON NITRIDE	3.00
B	RonaCare® Nicotinamide (Merck)	NIACINAMIDE	2.00
	RonaCare® MAP (Merck)	MAGNESIUM ASCORBYL PHOSPHATE	1.50
	Water, demineralized	AQUA	ad 100
	Complexing agent		q.s.
	Glycerol 85 % (Merck)	GLYCERIN	3.00
C	Rhodicare S (Rhodia)	XANTHAN GUM	0.60
	RonaCare® Cyclopeptide-5 (Merck)	AQUA, ALCOHOL, LECITHIN, ECTOIN, CYCLOTETRAPEPTIDE-24 AMINOCYCLOHEXANE CARBOXYLATE	2.00
	Preservatives		q.s.
	Fragrance	PARFUM	q.s.

Appearance: White lotion

pH (23 °C): 6.9

Viscosity (23 °C): 7,500 mPas (Brookfield DVII+ Pro, Helipath spindle B, 10 rpm)

Stability: 3 months at 40 °C, FTC (-5 °C to 40 °C), 5 °C, RT and 1 week at 50 °C

**Formulation 3** Ultra Smooth Yoghurt Shake – O/W lotion with Methoxyphenyl t-Butylphenyl Propanediol, Niacinamide and Magnesium Ascorbyl Phosphate.

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## **A Global and Powerful Approach to Circumvent the Harmful Effects of Blue Light and IR-A Irradiation on the Skin**

Valérie Bicard-Benhamou, Marina Lefort, Lilia Heider, Jutta zur Lage, Silke Hornung, Heike Hanau, Alexander Kielbassa, Christophe Carola, Joerg von Hagen  
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# A Global and Powerful Approach to Circumvent the Harmful Effects of Blue Light and IR-A Irradiation on the Skin

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**Keywords:** Blue light, HEVL, IR-A, human explant protection, biochemical markers, first-line defense, second-line defense

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## INTRODUCTION

Appropriate and controlled doses of UV, HEVL and IR may be beneficial for solving different skin problems; however, protection in all these ranges is needed. It often remains in mind that photoprotection is mainly a matter of UV protection. However, all solar radiation leads to the formation of radical oxygen species (ROS), and an excess of free radicals in the skin contributes to premature skin aging and wrinkling. Visible light, and especially high-energy visible light (HEVL, 400-500nm), notably triggers pro-inflammatory cytokines, the expression of different matrix metalloproteinases, and/or oxidation of proteins. All these markers play a role in accelerating skin aging (e.g. sagging skin, inflammation, wrinkles, and pigmentation disorders). In comparison, infrared (IR) radiation has the lowest energy level, but its contribution to the solar spectrum reaching the human skin is about 45 % [1]. Therefore, the biological impact on the skin may be strong [2]. IR light, and particularly IR-A (700-1400nm), induces significant free radicals in the dermis and diminishes the skin's antioxidant capacity [3]. Additionally, at high doses, harmful effects are reported [4]. IR radiation has been shown to alter the collagen content of the dermal extracellular matrix (ECM), not only by causing increased expression of the collagen-degrading enzymes MMPs, but also by decreasing *de novo* collagen synthesis [4].

There are already powerful solutions to achieve light protection beyond UV, notably in the HEVL and IR-A ranges, considering the first-line defense strategy that means preventing any radiation from penetrating the skin. To achieve this first-line defense protection appropriate combinations of specific titanium dioxide UV filters and mica-based functional fillers could be used [5,6]. In this work, we present a

complementary approach mainly targeting second defense line, meaning reducing damage that nevertheless may occur in the skin considering an over-exposure to HEVL (blue light) and/or IR-A radiations.

Cosmetic ingredients from different origins were selected: two ingredients known for their antioxidant properties: a synthetic one, Bis-ethylhexyl hydroxydimethoxy

## Abstract

### Research Objective

*The effect of solar radiation in the blue light or high energy visible light range, as well as in the longer wavelengths such as infrared, is an increasingly important topic of research. Indeed, the potential damage to the skin is a real issue for the skin. There is therefore a need to identify efficient cosmetic solutions enabling protection of the skin beyond UV range.*

### Experimental methods

*We present here facts showing the performance of different ingredients on human explants after irradiation with blue light and IR-A in 2 different ex-vivo studies. Ingredients of different origin were applied to human explants. An emulsion containing a titanium dioxide UV filter grade (first-line defense equivalent to a physical barrier) was tested, as well as*

*second-line defense ingredients: 2 antioxidants, the natural extract Phyllanthus Emblica fruit extract and the synthetic Bis-ethylhexyl hydroxydimethoxy benzylmalonate, and a natural cell protector Ectoin. These ingredients are described as second-line defense ingredients (equivalent to a biochemical protection barrier in the skin). Finally, the performance of the self-tanner dihydroxyacetone was also investigated. To assess the level of efficacy of the ingredients, different and representative biochemical markers were immunostained.*

### Main observations

*Our findings showed that all tested products had very good performance on several biochemical parameters and in both wavelengths ranges. Photoaging damage may therefore be slowed down and the onset of aging may be delayed.*

benzylmalonate (HDBM) and a natural extract, *Phyllanthus Emblica* Fruit Extract. Furthermore, we selected a natural cell protection agent: Ectoin. Validating the link between a first-line defense strategy and the impact on biochemical markers after a stress with HEVL/IR-A was also in focus. Therefore, a fourth ingredient, in the form of an emulsion containing a titanium dioxide UV filter grade (UV-TiO<sub>2</sub>/SiO<sub>2</sub>), was tested. Finally, we included a compound that may act on both the first and second defense line: the well-known self-tanner dihydroxyacetone (DHA). We tested DHA knowing that dark-colored materials absorb in the visible range, and that a certain photo-protection ability of topically applied DHA had been previously briefly reported in the UV-A and HEVL ranges [7-8]. We also investigated the longer wavelengths (infrared range). The assessment of the protective effect of the different ingredients against HEVL and IR-A radiation was carried out on living human skin explants. To obtain a comprehensive picture of the mechanism of action and performance of the different ingredients, multiple parameters were tested. Immunostaining of tropoelastin and MMP-1 was investigated for explants irradiated with IR-A and the expression of MMP-1, 8-OHdG, Opsin-3 and oxidized protein level were assessed for explants irradiated with HEVL.

## EXPERIMENTAL

### 1. Products tested

#### Ectoin (1% in water)

Ectoin was first discovered in *Ectothiorhodospira halochloris*, a halophilic bacterium that survives and grows in extreme conditions and high temperatures in salt lakes, saline soils, sea water and saline deserts. Meanwhile, other halophilic bacteria species, such as *Halomonas elongata*, have been found to generate Ectoin as a protective mechanism. Ectoin is a multi-talented natural ingredient [9-10] notably showing immune system protection, cell protection, super moisturizing: for example, it notably induces moisture accumulation in skin that is still detectable 7 days after application [35]. In addition, Ectoin protects against protein oxidation

induced by dehydration stress (27), anti-aging (e.g., roughness reduced by 53% and wrinkle volume reduced by 23% after 4 weeks treatment on the skin (36)) and has anti-pollution properties. Ectoin is an ingredient from Merck KGaA, Darmstadt, Germany.

#### *Phyllanthus Emblica* fruit extract (Embllica) (0.5% in water)

Embllica is obtained from *Phyllanthus Emblica officinalis*, also known as Indian gooseberry, a sustainable resource that is well known in Ayurvedic medicine. It has long-lasting antioxidant properties: a DDPH test showed how Embllica retains full antioxidant activity after 12 months at 45 °C, whereas other ingredients (vitamin C, rosemary extract, pine antioxidant, vitamin E) failed [11]. Embllica also shows excellent singlet oxygen/superoxide anion/hydroxyl radical quenching abilities (against <sup>1</sup>O<sub>2</sub>: IC50<sub>(Embllica)</sub> 61 µg/ml, IC50<sub>(Trolox)</sub> 84 µg/ml; against <sup>•</sup>O<sub>2</sub>: IC50<sub>(Embllica)</sub> 12 µg/ml, IC50<sub>(Vit C)</sub> 26 µg/ml, IC50<sub>(Trolox)</sub> 360 µg/ml), as well as strong metal-chelating properties in comparison with other chelators tested (for instance, vitamin C, pine antioxidant, green tea) [12,26]. Embllica fruit extract is an ingredient from Merck KGaA, Darmstadt, Germany.

#### Bis-ethylhexyl hydroxydimethoxy benzylmalonate (HDBM) (1% in ethanol)

It is a pure, stable, and transparent cosmetic oil that can be directly added to the oil phase of an emulsion and remains stable at higher temperatures (up to 80 °C) and over a broad pH range [4-8].

HDBM is a powerful and long-lasting antioxidant. It is notably as stable as α-tocopherol acetate and as active as ascorbic acid simultaneously (DPPH test). It has been proven to combine high efficacy with lasting stability in cosmetic emulsions. It can provide four equivalents of hydrogen for radical scavenging purposes (one phenolic, two benzylic, one malonic). The oxidation product of HDBM itself has antioxidant properties and its activity is therefore maintained over time [13].

As a result of oxidation, the single bond (HDBM) can be converted into a double

bond (HDBMox), which then possesses the ability to quench singlet oxygen, thus ideally completing the system [14]. HDBM is an ingredient from Merck KGaA, Darmstadt, Germany.

Titanium dioxide UV filter grade (INCI: titanium dioxide, silica) (acronym for the rest of the text UV-TiO<sub>2</sub>/SiO<sub>2</sub>) used in 3% o/w emulsion. UV-TiO<sub>2</sub>/SiO<sub>2</sub> is a 100% inorganic UV filter, transparent on the skin, with an efficient broad-spectrum efficacy in the UVA/B range. It also provides first-line defensive efficacy against HEVL, visible and IR radiation, as shown in previous experiments by Rozman *et al.* [6]. UV-TiO<sub>2</sub>/SiO<sub>2</sub> is an ingredient from Merck KGaA, Darmstadt, Germany.

#### Dihydroxyacetone (DHA) (2% in water)

DHA of high purity, is a self-tanning agent produced by a biotechnological process providing strong skin coloration efficacy. DHA reacts with the proteins and amino acids of the horny layer of the skin (Maillard reaction), in which resulting polymers have the well-known intense brown color. DHA is an ingredient from Merck KGaA, Darmstadt, Germany.

## 2. Experiments investigating HEVL impact on human skin explants

### Explant preparation

Human skin explants were prepared in an abdominoplasty from a 45-year-old Caucasian woman (skin phototype II). The explants were kept in survival in a specific culture medium (BIO-EC's Explants Medium) at 37 °C in a humid, 5% CO<sub>2</sub> atmosphere. Three explants per treatment were used for the experiments. On day 0, day 3 and day 4 (before blue light irradiation), the tested products were applied topically. The blank batch did not receive any treatment except the renewal of the culture medium. On day 4, the explants of the relevant batches were irradiated with the Solarbox<sup>®</sup> blue light using a dose of 84.96 J/cm<sup>2</sup> for 4 hours.

### Immunostaining of Opsin-3

Opsin-3 is expressed in several tissues, including skin. It serves as a sensor for blue light in melanocytes and therefore functions as a sensor for visible light pigmentation.

tion. Opsin-3 appears to be a new potential target for regulating melanogenesis [15].

Opsin-3 immunostaining was performed on paraffin sections with a polyclonal anti-Opsin-3 antibody (Novus Biologicals, ref. NBP2-55726) diluted at 1:50 in PBS-BSA 0.3 %-Tween 20 at 0.05 % for 1 hour at room temperature, using a Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP, a violet substrate of peroxidase (Vector, ref. SK-4600). The immunostaining was performed using an automated slide processing system (Autostainer, Dako) and assessed by microscopic observation. Three pictures were taken per explant, meaning 9 pictures were available for each treatment.

#### 8-OHdG immunostaining

8-hydroxydesoxyguanosine (8-OHdG) reflects the degree of oxidative damage to DNA and contributes to signs of premature skin aging [16]. 8-OHdG immunostaining was performed on paraffin sections with a monoclonal anti-8-OHdG antibody (Gentaur, ref. 50-MOG, clone N45-1) diluted at 1:100 in PBS-BSA 0.3 % at 0.05 % overnight at room temperature, using a Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP, a violet substrate of peroxidase (Vector, ref. SK-4600). The immunostaining was performed manually and assessed by microscopic observation, with semi-quantification by image analysis. Three pictures were taken per explant, meaning 9 pictures were available for each treatment.

#### Oxidized proteins immunostaining

Accumulation of damaged proteins is one of the most fundamental features of aging. Carbonylated protein content is the usual general biomarker of severe oxidative protein damage/inactivation. The oxidized proteins were stained on frozen sections after pre-incubation with DNPH (2,4-dinitrophenylhydrazine, Millipore, ref. 90448) and incubation with an anti-DNP antibody (Millipore, ref. 90451) diluted at 1:250 in PBS, BSA 0.3 % for 1 h at 37 °C, with a biotin/streptavidin-amplifying system and revealed with VIP, a violet substrate of peroxidase (Vector, ref. SK-4600). The immunostaining was assessed by microscopic observation. Three pictures

were taken per explant, meaning 9 pictures were available for each treatment.

#### MMP-1 immunostaining

Matrix metalloproteinases (MMPs) are peptidase enzymes responsible for the degradation of ECM components. Irradiation of human skin equivalents with HEVL induced production of MMP-1. HEVL exposure therefore contributes to signs of premature skin aging [17].

MMP-1 immunostaining was performed on paraffin sections with a polyclonal anti-MMP-1 antibody (Sigma, Ref M4696) diluted at 1:100 in PBS-BSA 0.3 %-Tween 20 at 0.05 % for 1 h at room temperature, using Vectastain Kit Vecot amplifier system avidin/biotin and revealed by VIP, a violet substrate of peroxidase (Vector Laboratories, ref SK-4600). The immunostaining was performed using an automated slide processing system (Autostainer, Dako) and assessed by microscopic observation. Three pictures were taken per explant, meaning 9 pictures were available for each treatment.

Known impacts of blue light exposure on these markers, as well as their desired modulation on treatment with the different products, are summarized in **Table I**.

#### Image analysis method

Image analyses were performed on all images in each batch, according to the following method using Cell<sup>^</sup>D software. For each batch of explants, the percentage of the region of interest covered by the staining (stained surface percentage) was determined by image analysis (see raw values in **Table II-VII**). We thus expressed all our results in percentage terms relative to control and/or irradiated control.

#### Statistical analysis

Statistical analysis was performed using Student t-test.

### 3. Experiments investigating infrared A light (IR-A) on human skin explants

#### Explant preparation

Human skin explants were prepared on an abdominoplasty from a 61-year-old Caucasian woman (Fitzpatrick skin pho-

totype II-III). The explants were kept alive in a specific culture medium (BIO-EC's Explants Medium) at 37 °C in a humid, 5 % CO<sub>2</sub> atmosphere. On day 0, day 3, day 4 (3 hours before IR irradiation), day 5 and day 6, the tested products were applied topically, followed by 10 minutes of drying. The blank batch did not receive any treatment except the renewal of the culture medium. The culture medium was half-renewed (1 ml/well) on day 3 and day 5. Irradiation of the explants was done using an infrared lamp (Dr Fischer 1000W, 235V 2500K; 760-3000nm), for 5040 seconds (720J/cm<sup>2</sup>) with an intensity of 143 mW/cm<sup>2</sup>. According to *Weiss et al.* [32], the NIR irradiance of the solar spectrum is 72 mW/cm<sup>2</sup>. So, the device is approximately twice as powerful as the sun: it takes half the time of the sun to deliver the same dose of NIR. The dose 720J/cm<sup>2</sup> was determined according to *Schroeder et al* [19]. According to *Barolet et al.* [33], this dose of 720J/cm<sup>2</sup> can be described as medium. It was delivered in 1 hour and 24 minutes, which corresponds to a solar exposure of 2 hours and 48 minutes.

To avoid temperature increases, and therefore heat damage known to occur in the IR-B and IR-C ranges [18], we focused on the IR-A range, and water filter and IR760 filter were also used to achieve the following IR range 700-1150 nm. The explants' temperature was measured before and after IR irradiation. The explants' temperature was maintained at 37 °C (max 39 °C) by a specially adapted refreshing system.

#### MMP-1 immunostaining

See above in the HEVL section. It is known from literature that the irradiation of human skin equivalents with infrared light induces production of MMP-1 [19].

#### Tropoelastin

Elastic fibers are found in the ECM of connective tissue, providing elasticity and resilience to tissues.

Due to extreme insolubility of elastin, we focused on its soluble precursor, tropoelastin.

Under a single and medium IR-A irradiation, elastic fibers are degraded and expres-

**Table I** Impact of blue light and IR light on literature-known biomarkers and envisioned modulation upon treatment with the tested products.

Marker	Model	Impact after HEVL	Impact after IR-A	Expected impact after HEVL and upon treatment with the different products vs irradiated control	Expected impact after IR-A and upon treatment with the different products vs irradiated control	Selected references
MMP-1	Reconstructed human epidermis (RHE)	Increase	Increase	Decrease	Decrease	(15), (21)
Carbonylated proteins	Ex vivo	Increase		Decrease		(23)
8-OHdG	E.g. ex vivo	Increase		Decrease		(14),(16)
Opsin-3	Normal human melanocytes (NHM)	Increase		Decrease		(13)
Tropoelastin			Decrease		Increase	(20)

sion of tropoelastin units is reduced [20]. Tropoelastin immunostaining was carried out on frozen sections with a monoclonal anti-tropoelastin antibody (Chemicon, ref MAB2503, clone 10B8) diluted at 1:200 in PBS-BSA 0,3% overnight at 4°C, using Vectastain Kit Vector amplifier system avidin/biotin and revealed by AF488 (Lifetechnologies, ref. A11001). The nuclei were counterstained by propidium iodide. The immunostaining was assessed by microscopic observation. Three pictures were taken per explant, meaning 9 pictures were available for each treatment.

Known impacts on IR-A exposure on these 2 markers, as well as their desired modulation on treatment with the different products, are summarized in *Table I*.

## RESULTS AND DISCUSSION

### 1. Results of the ex vivo study investigating HEVL impact on human skin explants

All raw data have been summarized in *Tables II-VII*.

#### Immunostaining of oxidized proteins

Oxidized proteins were stained in the papillary dermis, and the results showed the following outcome:

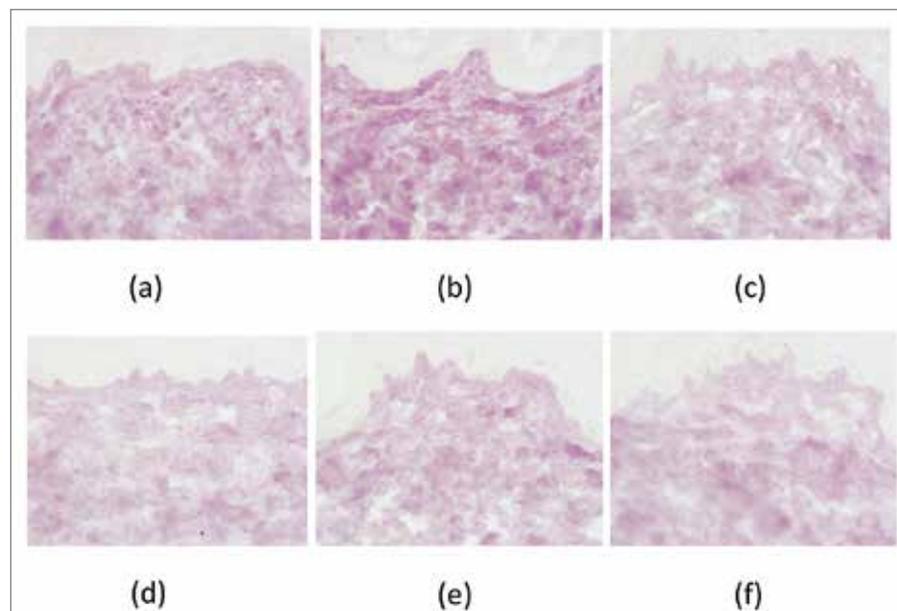
The blue light irradiation induced a visible and very significant increase of 95%\*\* ( $p < 0.01$ ) of oxidized protein formation in the papillary dermis relative to the control batch at day 5. Upon exposure of the human explants to HEVL, and application of the products, the intensity of the oxidized protein immunostaining was visibly and significantly reduced for all products vs the irradiated untreated control: the formula-

tion containing UV-TiO<sub>2</sub>/SiO<sub>2</sub> (-47%\*\*,  $p < 0.01$ ), Emblica (-44%\*\*,  $p < 0.01$ ), Ectoin (-59%\*\*,  $p < 0.01$ ) and DHA (-61%\*\*,  $p < 0.01$ ) (see *Figure 1* and raw data on *Table V*). In the case of HDBM (solubilized in ethanol), no comparison with irradiated ethanol control explant is available. However, the intensity of the oxidized protein immunostaining vs the irradiated control could be very significantly decreased (data not shown).

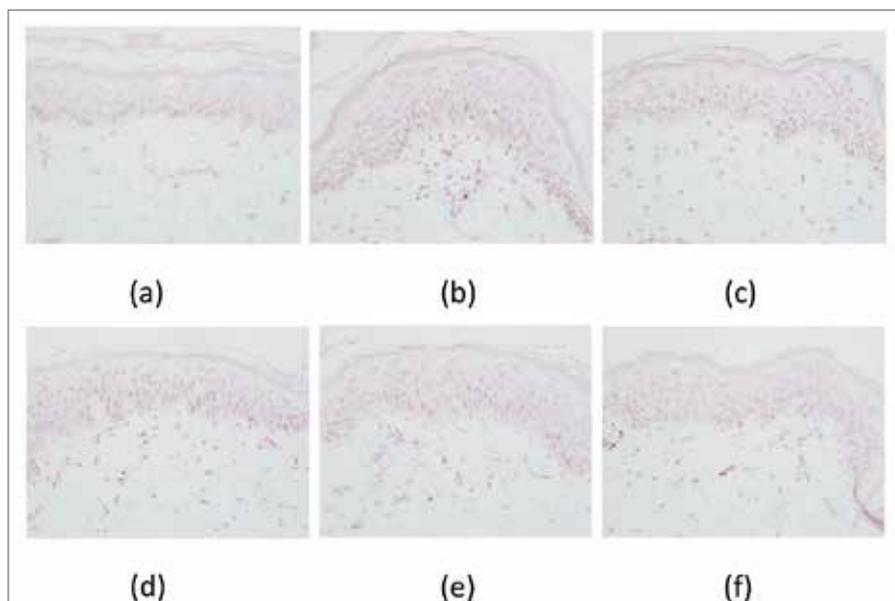
#### Immunostaining of 8-OHdG

Nuclear and mitochondrial DNA oxidation occur most readily in guanine residues owing to the high ionization potential of this base. 8-hydroxydesoxyguanosine (8-OHdG) is one of the predominant forms of free-radical-induced oxidative lesions in humans [16]. The interaction of hydroxyl radicals with the double bond at the C-8 position of the guanine base leads to the production of 8-OHdG. This stable oxidative modified DNA product has extensively been used to reflect the degree of oxidative damage to DNA [21].

After HEVL exposure, the formation of 8-OHdG is significantly increased by 37%\* ( $p < 0.05$ ) in the epidermis compared with the control batch at day 5. Upon exposure of the human explants to HEVL and application of the products,



**Figure 1** Immunostaining of oxidized proteins. Representative pictures at day 5. (a) control; (b) control + HEVL; (c) Ectoin + HEVL: -59%\*\* vs (b); (d) DHA + HEVL: -61%\*\* vs (b); (e) formulation cont. UV-TiO<sub>2</sub>/SiO<sub>2</sub> + HEVL: -47%\*\* vs (b); (f) Emblica + HEVL: -44%\*\* vs (b).



**Figure 2** Immunostaining of 8-OHdG. Representative pictures at day 5.

(a) control; (b) control + HEVL; (c) Ectoin + HEVL: n.s. reduction vs (b); (d) DHA + HEVL: n.s. reduction vs (b); (e) formulation cont. UV-TiO<sub>2</sub>/SiO<sub>2</sub> + HEVL: -13%\* vs (b); (f) Emblica + HEVL: -15%\* vs (b).

it could be observed that the 2 following products were able to decrease the levels of 8-OHdG compared with the irradiated control at a similar level: the formulation containing UV-TiO<sub>2</sub>/SiO<sub>2</sub> (-13%\*,  $p < 0.05$ ), Emblica (-15%\*,  $p < 0.05$ ). Ectoin did not show any impact on 8-OHdG levels vs the irradiated control. (**Figure 2** and **Table IV**). In the case of HDBM (solubilized in ethanol), no comparison with irradiated ethanol control explant was available. However, the intensity of 8-OHdG immunostaining vs the irradiated control could be significantly decreased (data not shown).

### Immunostaining of Opsin-3

Opsins are members of the guanine nucleotide-binding protein (G protein)-coupled receptor superfamily. In addition to the visual opsins, mammals possess several photoreceptive non-visual opsins that are expressed in extra-ocular tissues. This gene, Opsin-3, is expressed in several tissues, including the skin. It has been recently shown that melanocytes sense blue light and regulate pigmentation through Opsin-3 [15]. HEVL exposure induced a significant increase of 72%\*\* ( $p < 0.01$ ) in Opsin-3 expression in the epidermis vs control at day 5. The strongest decrease in Opsin-3 after irradiating the explants with

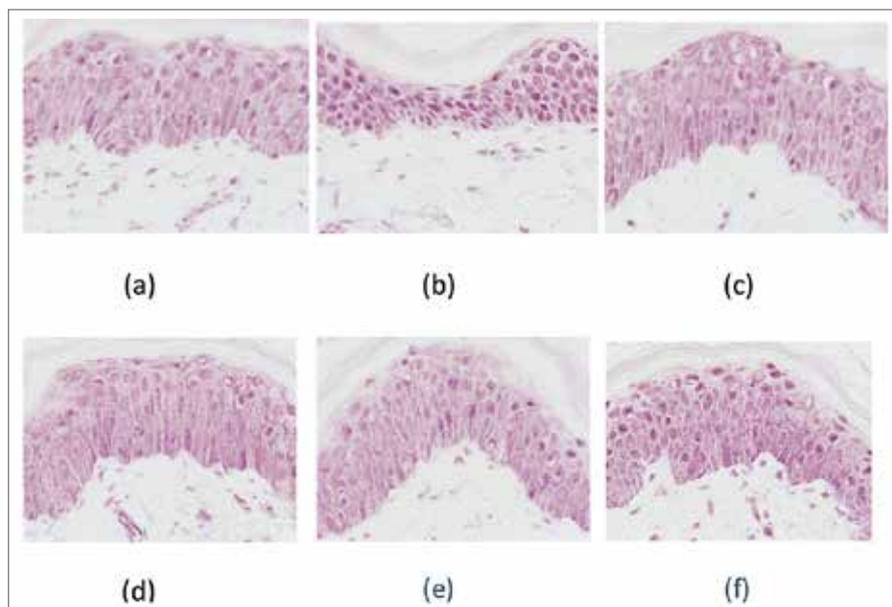
HEVL and treating them with the products was observed for the emulsion containing 3% UV-TiO<sub>2</sub>/SiO<sub>2</sub> (-37%\*\* vs irradiated control;  $p < 0.01$ ). The reduction in immunostaining intensity is significantly visible as shown in **Figure 3**. With Ectoin treatment and HEVL irradiation, a decrease in the Opsin-3 level of 24%\*\* ( $p < 0.01$ ) vs irradiated control was observed. DHA

provided a decrease in immunostaining of 23%# ( $p < 0.1$ ) vs irradiated control and Emblica, a decrease of -23%\* ( $p < 0.01$ ) vs irradiated control.

The synthetic antioxidant (HDBM) did not show any significant decrease vs irradiated control in this test. See also raw data in **Table III**.

### Immunostaining of MMP-1

Matrix metalloproteinases (MMPs) are a family of peptidase enzymes responsible for the degradation of extracellular matrix components, including collagen I, gelatin, fibronectin, laminin and proteoglycan. HEVL exposure notably induces MMP-1 expression, and therefore contributes to signs of premature skin aging [17]. HEVL irradiation induced a significant increase of 77%\* ( $p < 0.05$ ) of MMP-1 expression in the epidermis vs control at day 5. The intensity of MMP-1 immunostaining is visibly and significantly decreased for all products vs the irradiated control. Nonetheless, HDBM showed the strongest performance and was able to decrease by 31%\*\* ( $p < 0.01$ ) MMP-1 expression in the epidermis in comparison with the irradiated ethanol control and corresponding to -36%\*\* vs irradiated control. The other products also decreased MMP-1 levels vs irradiated control: the formulation

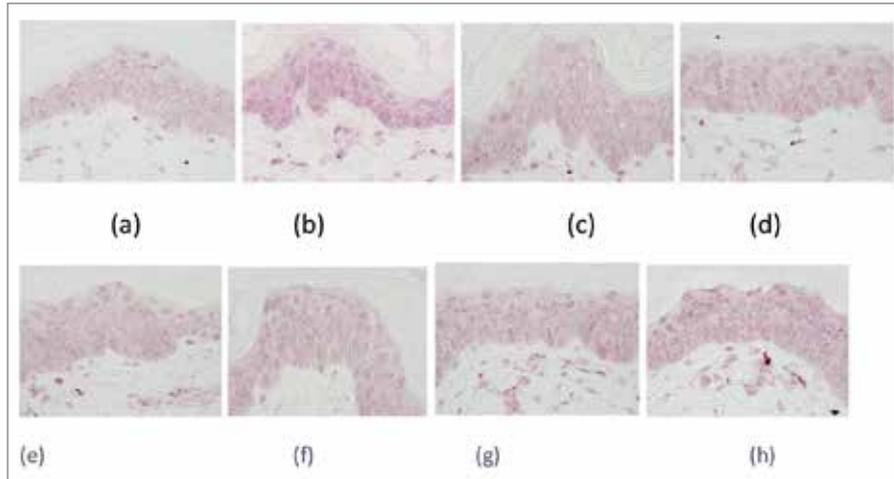


**Figure 3** Immunostaining of Opsin-3. Representative pictures at day 5.

(a) control; (b) control + HEVL; (c) Ectoin + HEVL: -24%\*\* vs (b); (d) DHA + HEVL: -23%# vs (b); (e) formulation cont. UV-TiO<sub>2</sub>/SiO<sub>2</sub> + HEVL: -37%\*\* vs (b); (f) Emblica + HEVL: -23% vs (b).

**Table II** Surface percentage positive to MMP-1 in the epidermis – HEVL Study

MMP-1	Irradiated explant (day 5)	Irradiated explant treated with ethanol (day 5)	Irradiated explant treated with Ectoin (day 5)	Irradiated explant treated with HDBM (day 5)	Irradiated explant treated with Emblica (day 5)	Irradiated explant treated with DHA (day5)	Irradiated explant treated with the formulation cont. UV-TiO <sub>2</sub> -SiO <sub>2</sub> (day5)
Average	54.6	50.4	43.8	34.8	41.9	41.8	43.6
SD	6.4	11.5	6.4	8.8	6.0	3.3	2.5



**Figure 4** Immunostaining of MMP-1. Representative pictures at day 5.

(a) control; (b) control + HEVL; (c) Ectoin + HEVL: -20%<sup>\*\*</sup> vs (b); (d) DHA + HEVL: -23%<sup>\*</sup> vs (b); (e) formulation cont. UV-TiO<sub>2</sub>/SiO<sub>2</sub> + HEVL:-20%<sup>\*\*</sup> vs (b); (f) Emblica + HEVL: -23%<sup>\*\*</sup> vs (b); (g) HDBM + HEVL: -31%<sup>\*\*</sup> vs (h), (h) control + ethanol+ HEVL.

containing 3% UV-TiO<sub>2</sub>/SiO<sub>2</sub> (-20%<sup>\*\*</sup>;  $p < 0.01$ ), Emblica (-23%<sup>\*\*</sup>;  $p < 0.01$ ), Ectoin (-20%<sup>\*\*</sup>;  $p < 0.01$ ) and DHA (-23%<sup>\*</sup>;  $p < 0.05$ ). See pictures in **Figure 4** and raw data in **Table II**.

## 2. Results of the study investigating IR-A impact on human skin explants

All raw data have been summarized in **Tables VI** and **VII**.

### Immunostaining of MMP-1

Matrix metalloproteinase-1 (MMP-1) is an enzyme also known as interstitial collagenase and fibroblast collagenase. It was previously shown that IR-A radiation elicits a retrograde signaling response, which is initiated in the mitochondria through generation of reactive oxygen species (ROS) that originate from the mitochondrial electron transport chain. One major biological consequence of this retrograde signaling response is the increased expression of matrix metalloproteinase (MMP-1) [22].

An IR-A irradiation of the explants induced a clear increase in MMP-1 expression in

the epidermis (+208%<sup>\*\*</sup>,  $p < 0.01$ ). Upon IR-A exposure, two products in particular showed good ability to decrease MMP-1 levels vs irradiated control: DHA (-67%<sup>\*\*</sup>;  $p < 0.01$ ) and the formulation

containing 3% UV-TiO<sub>2</sub>/SiO<sub>2</sub> (-65%<sup>\*\*</sup>;  $p < 0.01$ ). The natural antioxidant also showed a good performance vs irradiated control: Emblica (-48%<sup>\*\*</sup>;  $p < 0.01$ ). Finally, Ectoin moderately decreased MMP-1 expression in IR-A irradiated explants (-22%<sup>\*</sup>,  $p < 0.05$ ). Pictures are shown in **Figure 5** and raw data in **Table VI**. In the case of HDBM (solubilized in ethanol), no comparison with irradiated ethanol control explant was available. However, the intensity of MMP-1 immunostaining vs the irradiated control could be very significantly decreased (data not shown).

### Immunostaining of Tropoelastin

Elastin is the major component (90%) of the elastic fibers [23-24]. It is a protein of the connective tissue responsible for the elastic properties of the skin. It is initially synthesized as its precursor, tropoelastin. The impact of infrared radiation on tropoelastin expression at mRNA and protein levels has been investigated [18, 25].

The staining of tropoelastin in the papillary dermis showed the following results: An IR-A irradiation of the explants induced a significant decrease of 45%<sup>\*\*</sup> ( $p < 0.01$ ) in tropoelastin expression in the papillary dermis at day 7.

**Table III** Surface percentage positive to Opsin-3 in the epidermis – HEVL Study

Opsin-3	Irradiated explant (day 5)	Irradiated explant treated with Ectoin (day 5)	Irradiated explant treated with Emblica (day 5)	Irradiated explant treated with DHA (day5)	Irradiated explant treated with the formulation cont. UV-TiO <sub>2</sub> -SiO <sub>2</sub> (day5)
Average	21.8	16.6	16.7	16.8	13.8
SD	4.4	1.4	3.7	6.4	3.6

**Table IV** Surface percentage positive to 8-OHdG in the epidermis – HEVL study

8-OHdG	Irradiated explant (day 5)	Irradiated explant treated with Ectoin (day 5)	Irradiated explant treated with Emblica (day 5)	Irradiated explant treated with DHA (day 5)	Irradiated explant treated with the formulation cont. UV-TiO <sub>2</sub> -SiO <sub>2</sub> (day 5)
Average	26.9	25.9	22.8	25.6	23.4
SD	3.3	2.5	3.0	3.5	2.7

The formulation containing 3% UV-TiO<sub>2</sub>/SiO<sub>2</sub> induced the strongest, and very significant, tropoelastin increase of 116%<sup>\*\*</sup> ( $p < 0.01$ ) relative to the irradiated control. Three other ingredients also showed very good ability to increase tropoelastin expression: Emblica (+79%<sup>\*\*</sup>,  $p < 0.01$ ), HDBM (+86%<sup>\*\*</sup> vs irradiated ethanol control,  $p < 0.01$ ; +63%<sup>\*\*</sup> vs irradiated control,  $p < 0.01$ ), DHA (+57%<sup>\*\*</sup>;  $p < 0.01$ ), and finally Ectoin (+36%\*,  $p < 0.05$ ) in a more moderate manner. Pictures are shown in **Figure 6** and raw data in **Table VII**.

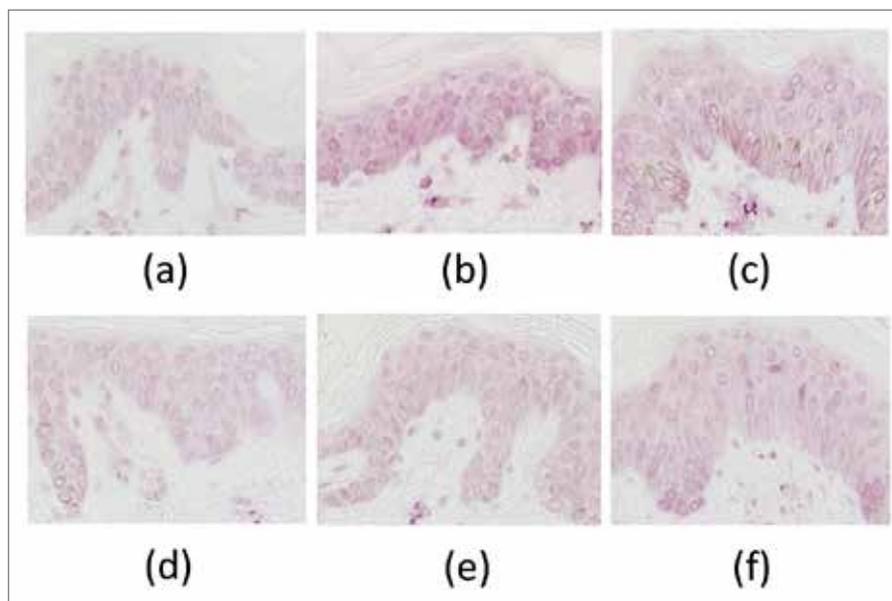
### 3. Discussion of both studies

#### UV-TiO<sub>2</sub>/SiO<sub>2</sub>

In both studies investigating the impact of HEVL and IR-A radiation, all the biomarkers tested were strongly and significantly impacted in a positive manner relative to the non-treated irradiated control explants (control + HEVL or control + IR-A). UV-TiO<sub>2</sub>/SiO<sub>2</sub> (INCI titanium dioxide, silica) formulated at 3% in an o/w emulsion is an inorganic material that prevents/minimizes free radical formation by absorbing, reflecting, and scattering properties of solar radiation, thus acting as first-line defense, and *de facto* provides a physical barrier. Radiation penetrating the skin is highly reduced. The studied biomarkers are known to be modulated by exposure to IR-A and HEV light (**Table I**).

In fact, the very limited amount of radiation reaching the skin after topical application of the emulsion containing UV-TiO<sub>2</sub>/SiO<sub>2</sub> notably explains the positive modulation of the biomarkers compared with irradiated control explants. The base emulsion used as placebo has been investigated in previous transmission/reflection/absorption measurements in both HEVL and IR-A ranges and showed no protective effect in both wavelength ranges compared with the same emulsion but containing UV-TiO<sub>2</sub>/SiO<sub>2</sub> [6]. We therefore made the assumption that this placebo emulsion would not provide any protective effect in the current *ex vivo* studies.

We can conclude that a strong explant protection may be achieved at a multi-parameter level for both wavelength ranges. The formulation containing 3% UV-TiO<sub>2</sub>/SiO<sub>2</sub> may



**Figure 5** Immunostaining of MMP-1. Representative pictures at day 5.

(a) control; (b) control + IR-A; (c) Ectoin + IR-A: -22%\* vs (b); (d) DHA + IR-A: -67%<sup>\*\*</sup> vs (b); (e) formulation cont. UV-TiO<sub>2</sub>/SiO<sub>2</sub> + IR-A: -65%<sup>\*\*</sup> vs (b); (f) Emblica + IR-A: -48%<sup>\*\*</sup> vs (b).

**Table V** Surface percentage positive to oxidized proteins in the dermis – HEVL study

Oxidized proteins	Irradiated explant (day 5)	Irradiated explant treated with Ectoin (day 5)	Irradiated explant treated with HDBM (day 5)	Irradiated explant treated with Emblica (day 5)	Irradiated explant treated with DHA (day 5)	Irradiated explant treated with the formulation cont. UV-TiO <sub>2</sub> -SiO <sub>2</sub> (day 5)
Average	71.3	29.3	41.3	39.5	28.0	37.7
SD	4.3	6.1	7.8	8.6	5.4	4.7

**Table VI** Surface percentage positive to MMP-1 in the epidermis – IR-A study

MMP-1	Irradiated explant (day 5)	Irradiated explant treated with Ectoin (day 5)	Irradiated explant treated with Emblica (day 5)	Irradiated explant treated with DHA (day 5)	Irradiated explant treated with the formulation cont. UV-TiO <sub>2</sub> -SiO <sub>2</sub> (day 5)
Average	25.3	19.7	13.1	8.4	8.9
SD	4.5	2.9	3.2	0.9	2.3

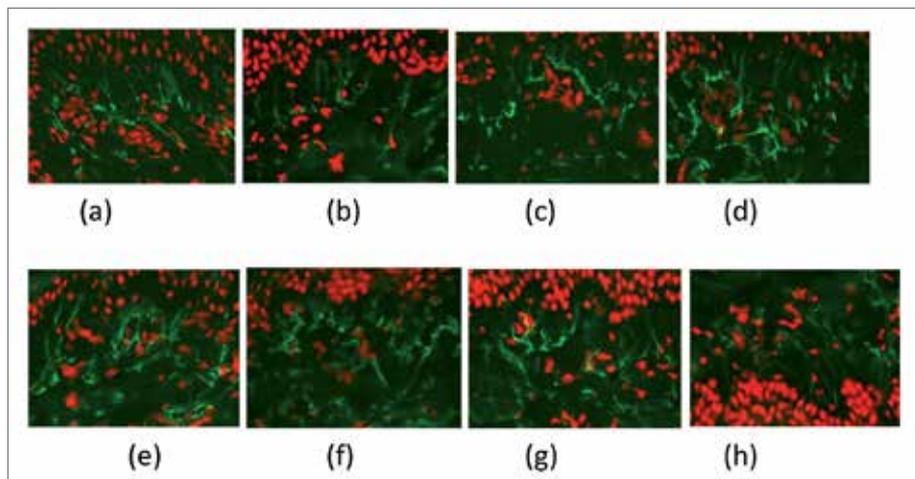
notably help to reduce the risk of blue-light-induced pigmentation (see Opsin-3 results), and in the case of the explants exposed to IR-A light, we could show a complete protection of tropoelastin (+116% vs control + IR-A) and thus a preservation of elastic fibers in the ECM. Knowing that HEVL and IR-A radiation both induce ROS formation, the use of the formulation containing 3% UV-TiO<sub>2</sub>/SiO<sub>2</sub> may minimize oxidative damage and therefore prevent premature aging.

These results also confirm previous investigations, which showed, by means of

transmission data measurements, how specific titanium dioxide UV filters prevent radiation from penetrating the skin [6]. In the light of our new results, we were able to make a link between first-line defense (physical barrier) and second-line defense (protection of biochemical endpoints).

#### HDBM

In the case of the HEVL study, we show the results for the MMP-1 biomarker vs irradiated ethanol control: -31%<sup>\*\*</sup>. For the other oxidized proteins and 8-OHdG biomarkers, there is no compar-



**Figure 6** Immunostaining of tropoelastin. Representative pictures at day 7.

(a) control; (b) control + HEVL; (c) Ectoin + HEVL: +36%\* vs (b); (d) DHA + HEVL: +57%\*\* vs (b); (e) formulation cont. UV-TiO<sub>2</sub>/SiO<sub>2</sub> + HEVL: +116%\*\* vs (b); (f) Emblica + HEVL: +79%\*\* vs (b); (g) HDBM + HEVL: +86%\*\* vs (h), (h) control+ ethanol+ IR-A.

ison available vs the irradiated ethanol control. Nonetheless the comparison vs irradiated control showed good results (data not shown).

In the IR-A study, we show the results for tropoelastin indicating positive changes from treatment with HDBM and after IR-A exposure vs control+ethanol+IR-A: +86%\*\* . For MMP-1, there is no comparison available vs the irradiated ethanol control. Nonetheless, the comparison vs irradiated control explant showed good results (data not shown).

As stated above, IR-A and HEVL irradiations are oxidative processes, and we can explain the performance of HDBM by its strong radical neutralization power, as depicted in **Figure 7** and as described in the experimental section. Oxidative damage after HEVL and IR-A irradiation is therefore minimized, and human explants may be protected against premature aging.

#### EMBLICA

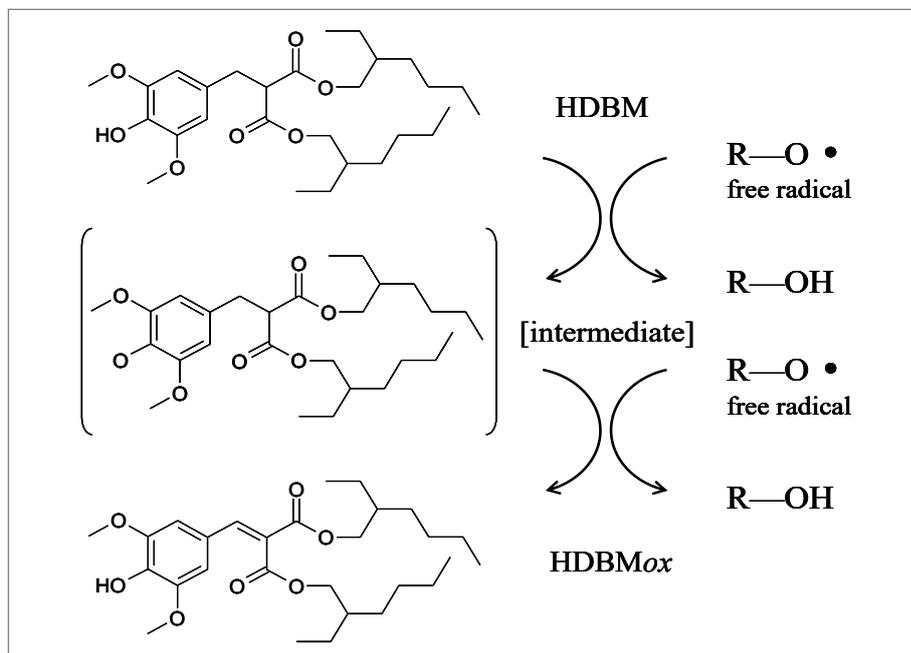
In the case of the HEVL study, four biomarkers were positively impacted by explant

treatment with the extract Emblica and after blue light irradiation vs irradiated control explant: the oxidized proteins (-44%\*\*), MMP-1 (-23%\*\*), 8-OHdG (-15%\*) and Opsin-3 (-23%\*).

Investigated biomarkers indicate positive changes from explant treatment with Emblica and after IR-A exposure vs irradiated control explant: (-48%\*\* for MMP-1 and +79%\*\* for tropoelastin).

Emblica was previously shown to decrease MMP-1 levels at an *in vitro* level, and therefore to protect most of the components of the extracellular matrix (ECM) [26]. This observation is further supported by our current results showing that Emblica is able to decrease MMP-1 expression in the presence of IR-A irradiation stress.

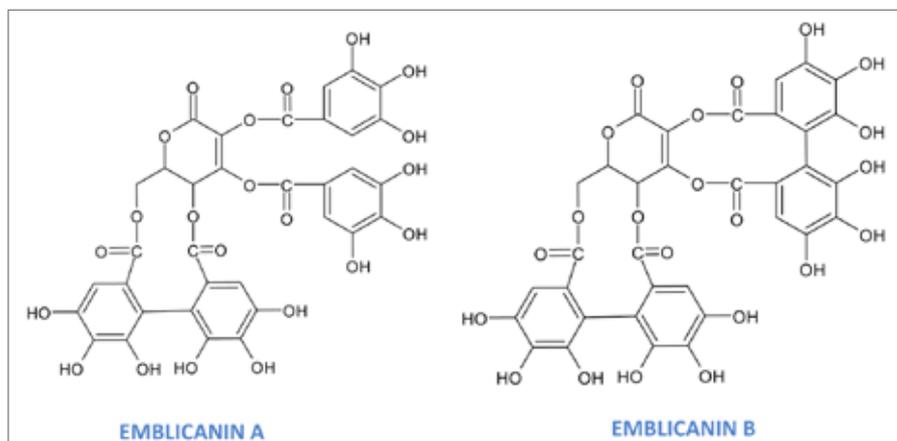
Emblica is a naturally powerful antioxidant, and its performance in these studies could



**Figure 7** Structure and mechanism of action of HDBM.

**Table VII** Surface percentage positive to tropoelastin in the papillary dermis – IR-A study

Tropoelastin	Irradiated explant (day 7)	Irradiated explant treated with ethanol (day 7)	Irradiated explant treated with Ectoin (day 7)	Irradiated explant treated with HDBM (day 7)	Irradiated explant treated with Emblica (day 7)	Irradiated explant treated with DHA (day 7)	Irradiated explant treated with the formulation cont. UV-TiO <sub>2</sub> -SiO <sub>2</sub> (day5)
Average	6.1	5.3	8.2	9.9	10.9	9.5	13.1
SD	1.4	1.5	1.7	2.8	2.7	2.2	2.2



**Figure 8** Main constituents of Emblica extract.

be largely explained by the broad antioxidative cascade of Emblicanin A and Emblicanin B, the main constituents of Emblica extract (see structures in **Figure 8**). Given that formation of free radicals can be reduced, DNA damage, as well as premature aging, may be minimized. Considering the results in the IR-A study, we conclude that Emblica can protect elastic fibers and preserve the integrity of the ECM.

It is interesting to note that, despite their structural differences, the two antioxidants (HDBM and Emblica) showed similar performance profiles and positively modulated the same biomarkers connected with oxidative processes, albeit to differing degrees.

### ECTOIN

In the case of the HEVL study, three biomarkers were positively impacted by treatment with Ectoin after blue light irradiation vs control + HEVL: the oxidized proteins (-59%\*\*,  $p < 0.01$ ), MMP-1 (-20%\*\*,  $p < 0.01$ ) and Opsin-3 (-24%\*\*,  $p < 0.01$ ).

The results obtained in oxidized proteins nicely confirm earlier results: Ectoin was indeed shown to reduce the carbonyl score and carbonylated protein expression in HaCat cells and human skin explants after exposure to HEVL [27].

Concerning MMP-1, earlier research also showed the photoprotective effect of Ectoin in the shorter wavelength range. Indeed, this substance could decrease MMP-1 expression in keratinocytes after exposure to UV-B [28].

Finally, we consider Opsin-3 modulation. This marker has recently attracted growing attention in cosmetic research. Indeed, *Regazetti et al* [15] showed that blue light stimulated melanogenesis by acting directly on melanocytes and via the Opsin-3 receptor.

In our experiments, the intensity of Opsin-3 immunostaining was significantly increased in the control explant exposed to blue light, which fits with the experimental observations of *Regazetti et al* [15]. The explant treated with Ectoin and exposed to HEVL showed a visible and significant reduction in Opsin-3 immunostaining intensity.

Taking the results of *Regazetti et al* linking Opsin-3 up-regulation and melanogenesis into account, Ectoin may help reduce the risk of blue light-induced pigmentation.

In the case of the IR-A study, both investigated biomarkers were positively impacted by treatment with Ectoin and after IR-A exposure vs control + IR-A. (-22%\*,  $p < 0.05$ ) for MMP-1 and +36%\*\* ( $p < 0.01$ ) for tropoelastin. To our knowledge, we show here for the first time that Ectoin also possesses a certain level of photoprotection in the longer wavelengths (IR-A).

A very interesting aspect of these results is the finding that Ectoin, which does not have any antioxidant properties, is able to circumvent skin explant damage associated with oxidative processes in the blue light and IR-A ranges. Further

research is needed and is currently under way to fully understand these results and the specific mode of action and role of Ectoin.

### DIHYDROXYACETONE

Within the HEVL study, three biomarkers were positively impacted by explant treatment with DHA and blue light irradiation vs irradiated control explants: the oxidized proteins (-61%\*\*,  $p < 0.01$ ) and Opsin-3 (-23%#,  $p < 0.1$ ).

DHA is a well-established self-tanner in the cosmetic industry [29]. DHA reacts with proteins and amino acids in the horny layer of the skin following the well-known Maillard reaction, in which the resulting polymers (melanoidins) give the skin a brownish hue *via* a reaction route which has not yet been fully clarified. This reaction is complete after approximately 4-6 hours. The tan achieved in this way cannot be washed off and is removed only with normal skin desquamation.

DHA also shows some moderate photoprotective properties. In the UV-B range, topically applied DHA offers a modest SPF of 2-3 in humans lasting from days to weeks (given that it is bound to the skin) [30]. The melanoidins (brown pigments) that are formed after topical application of DHA have been shown to exhibit absorption properties similar to those of melanin and provide moderate sunlight protection. Moreover, topical application of DHA gives the skin a certain photoprotection in the UV-A range [8, 31], as well as in the HEVL range. Our results confirm the earlier observations made by Johnson [7].

Nonetheless, to the best of our knowledge no data on the infrared range have previously been published, and this led us to investigate the impact of topically applied DHA in this range.

The biomarkers investigated indicate positive changes from explant treatment with DHA and after IR-A exposure vs irradiated control explant: -67%\*\* ( $p < 0.01$ ) for MMP-1 and +57%\*\* ( $p < 0.01$ ) for tropoelastin. This unexpected outcome led

us to measure the IR absorption spectrum of a blend of DHA ( $10^{-3}$ M) mixed with the amino acid lysine ( $10^{-3}$ M) in glycerol to better understand these results. This blend mimicked the Maillard reaction on the skin (skin liquid model [32]) and we could observe a clear absorption in the IR-A range (results not shown). It is nonetheless important to mention that this absorption is very low in comparison with common UV filter absorptions. These results offered the first hints to explain the ability of DHA to mitigate damage caused to human skin explants by IR-A irradiation. Further research is currently under way to fully understand the mechanism of action in these wavelengths.

Topical application of DHA works at a multi-parameter level for both HEVL and IR-A ranges and minimizes oxidative damage. DHA may help reduce the risk of blue-light-induced pigmentation and preserve components and integrity of the ECM, such as collagen and elastic fibers.

## CONCLUSION

This paper summarizes results investigating the effects of several different cosmetic ingredients on human skin explants after exposure to HEV and near infrared A (IR-A) light. Ingredients with different origins and different mechanisms of action were selected: two strong antioxidants, one of synthetic origin (HDBM), the second of natural origin (Emblica). A natural substance with cell-protecting properties but without any antioxidant properties was evaluated (Ectoin). Understanding the link between a first-line defense strategy and second-line biochemical defense strategy after stress with HEVL/IR-A was also investigated. Therefore, an emulsion containing a specific titanium dioxide UV filter providing a physical barrier to IR-A and HEVL rays was tested. Finally, we investigated the well-known self-tanner dihydroxyacetone (DHA). Different biochemical markers known to be modulated after an exposure to HEVL and/or IR-A were investigated.

After IR-A and HEVL irradiation, the formulation containing the selected titanium dioxide UV filter showed excellent protec-

tion of the human explants. All evaluated biochemical markers were modulated in a positive manner compared with the irradiated control explants. Therefore, the link between first-line defense (shown in earlier investigations) and second-line defense, that is, the impact on biochemical markers, could be validated. The antioxidants HDBM and the natural extract Emblica also showed excellent performance in relation to the tested biomarkers. It is interesting to note that Ectoin, which neither provides a physical barrier nor is an antioxidant, helps to mitigate damage to skin explants after different irradiation stresses. Finally, DHA showed very good results in both studies. Further research is continuing to get a full picture of the underlying mechanism of action in the case of the infrared study, which, to the best of our knowledge, showed for the first time the protective potential of DHA in the NIR range.

These results show how the tested products work on a multiple biochemical parameter level and help to minimize damage to human explants in a broad wavelength band of the solar spectrum.

Natural sunlight is the main source of HEVL and IR-A radiation and determines the probability and frequency of exposure. Natural sunlight is essential to life; however, human skin needs to be protected. The tested compounds are complementary for protection in both IR and visible light ranges at the cellular level. In line with consumer needs, a large choice of ingredients is now available, used either alone or possibly in different combinations to minimize the negative effects of HEVL and IR-A radiation on the skin: for instance, a first-line defense product (formulation containing UV-TiO<sub>2</sub>/SiO<sub>2</sub>) associated with second-line defense products (HDBM, Emblica, Ectoin, or even dihydroxyacetone).

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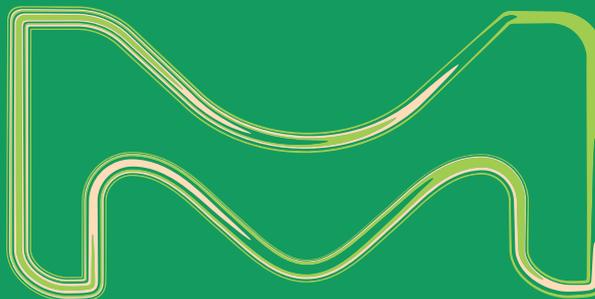
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