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Anti-pollution

Lievito di pichia

Fungo Reishi

CEC EDITORE
COSMETIC TECHNOLOGY®
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[TRANSLATION]

Nicomenthyl®

Transcutaneous niacin delivery and anti-pollution, *detox*, anti-oxidant efficacy

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Summary

The skin barrier biochemical mechanisms responsible for defence against endogenous and exogenous damaging agents have been widely researched (1-3). These studies evidenced the fundamental role played by niacin (nicotinic acid or vitamin B3), a precursor of the coenzyme NAD^+ (nicotinamide adenine dinucleotide). NAD+ is essential to all cellular processes involved in immune response and repairing DNA damage caused by UV radiation or aggressive chemicals. Niacin deficiency and consequent lack of NAD^+ in keratinocytes causes intracellular incidents which can lead to lipid peroxidation, oxidative stress of the cell membranes, premature skin ageing, erythema and skin irritation, and eventually more serious consequences, including unrepaired DNA damage, disruption of epidermal barrier integrity, mutagenesis, immune suppression, actinic keratosis and skin cancers. The effects of menthyl nicotinate, a lipophilic derivative of niacin designed to deliver niacin to skin without causing irritation or excessive vasodilation, have been studied through in vitro testing on human keratinocyte cultures, to evaluate its anti-oxidant, anti-pollution, detox, protective efficacy against four damaging agents (UV radiation, oxidizing agents, urban dust and synthetic smoke). Menthyl nicotinate is capable of rapidly penetrating the skin barrier, hydrolysing on contact with skin esterases, releasing niacin in the underlying cutaneous layers and so activating NAD^+ defence and repair activities. All tests indicate that menthyl nicotinate enhances cellular metabolism, protection and barrier function in skin against all four damaging agents. These scientific results are of great interest for cosmetic applications to sustain efficacy claims as anti-pollution, anti-aging, anti-free radicals, sun protection, and hair loss prevention.

Introduction

The present study aimed to characterize and investigate in detail some functional properties of menthyl nicotinate (**Figure 1, Table 1**) using a set of *in vitro* tests. Its specific characteristics that were assessed in a biological experimental model were its anti-oxidant, anti-pollution, detox and overall protective efficacy against some damaging agents, including urban polluting agents, cigarette smoke, oxidizing agents and UV radiation. This was done by choosing a set of *biomarkers* that may be assessed through fast protocols and whose biological meaning is universally acknowledged. Specifically, the protective efficacy was assessed by evaluating some basic parameters, such as cell viability and cell metabolism (**4, 5**); the oxidative damage was investigated by dosing malondialdehyde (MDA) (**6**), one of the main products of lipid peroxidation; and the detox action was investigated by measuring the activity of the glutathione S-transferase (**GST**) (**7**), one of the key enzymes for inactivating harmful xenobiotic agents. To better define the *performance* of the active ingredient, its efficacy against the damaging agents was investigated under acute and repeated exposure conditions.

Mechanism of action

After menthyl nicotinate has passed the stratum corneum, it undergoes hydrolysis and splits into its two original components: niacin and menthol. The latter causes the pleasant cooling sensation that may be experienced already a few minutes after application. Niacin, also known as vitamin B3 or vitamin PP (*Pellagra Preventive*) is a water-soluble vitamin belonging to group B. Once it has crossed the cell membrane of keratinocytes, it triggers a complex cascade of biochemical reactions that lead to the synthesis of one of the most important cellular coenzymes, NAD^+ (nicotinamide adenine dinucleotide). NAD^+ has a pivotal role, interacting with hundreds of enzymes involved in cell oxidative metabolism, in the Krebs cycle,

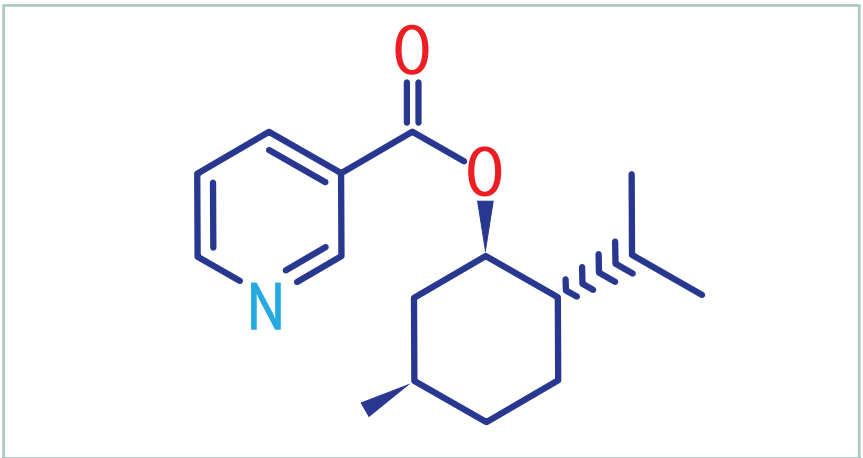

Figure 1 – Molecular structure of menthyl nicotinate.

Molecular weight	261.36
Molecular formula	$C_{16}H_{23}NO_2$
CAS number	40594-65-8
EINECS number	254-991-1
INCI name	Menthyl Nicotinate
REACH number	01-2120770053-62-0000
Appearance	Liquid
Colour	Transparent, colourless
APHA (Hazen)	≤20
Characteristic odour	Nearly odourless
Purity (GC-MS) (%)	>99.00
Relative density at 20°C	1.031
Solubility	Insoluble in water, soluble in alcohols, polar oils, esters
Boiling point (atmospheric pressure)	292.23°C
Melting point	< -20°C
Vapor pressure (at 20°C)	10 Pa
Partition coefficient (n-octanol/water) LogPow	5.09 at 25°C
Surface tension	36.19 mN/m at 22°C
Refractive index (Abbe Refractometer; 589 nm)	1.5060–1.5074
% of use in products	Face products: 0.5 – 1% Body products: 0.5 – 3%
Safety	Skin: not irritating, not sensitizing Not mutagenic
Shelf life: > 36 months. Thermostable. Does not require adding any preservative or antioxidizing agent. Does not require any special storage conditions.	

Table 1 – Technical features of the active ingredient

in the lipids oxidation mechanism, in glycolysis etc. Its reduced form, NADH, donates electrons to oxygen and is the main source of ATP (adenosine triphosphate), the molecule that provides temporary storage of the energy coming from the cellular respiration, and is therefore regarded as the "energy currency molecule" of the organism.

NAD^+ also plays a pivotal role as a substrate in enzymatic reactions involving protein modifications (transcribing DNA information into a complementary RNA molecule, regulating calcium homeostasis or DNA repair mechanisms). In fact, it is a substrate of poly (ADP-ribose) polymerases (PARP), a family of enzymes that are essential in activating defence mechanisms against the destructive effects of oxidizing agents such as radiations, free radicals, *reactive oxygen species* (ROS) and *reactive carbonyl species* (RCS). NAD^+ is also a substrate of sirtuins, or Sir2 proteins, a group of proteins possessing enzymatic activity that are involved in skin aging, transcription regulation, apoptosis, stress resistance and may affect energetic efficiency and vigilance under low calorie intake conditions.

After a toxic insult, especially when the epithelial defence mechanisms are weakened because of a lacking supply of niacin/NAD^+, leading to inactivation of PARP and sirtuins, intracellular events may occur, including lipid peroxidation, cell membranes oxidation, blockage of ion pumps and ATP synthesis inhibition that may lead to early skin aging, loss of skin barrier integrity, erythema, irritation and, in extreme cases, to DNA damage, mutations, immunosuppression and skin tumours (**1**).

Recent studies have shown, in fact, that low-level niacin keratinocytes are much more sensitive to UV exposure due to the accumulation of the ROS and RCS resulting from UV-induced harm and the consequent disruption of the previously cited skin defence and DNA repair enzymatic processes. This may even lead to developing epidermal hyperplasia and hyperkeratosis, actinic keratosis and squamous cell carcinoma (**2**).

This work provides a first preview of how the active ingredient, a lipophilic derivate of niacin, after passing rapidly through the skin barrier thanks to its lipophilia, releases niacin without inducing any hyperaemia or irritation. This prompts the aforementioned biochemical processes, giving the active ingredient a significant protective, antioxidizing, repairing and detox efficacy against a wide and heterogenous range of endogenous and environmental stress factors.

Materials and methods

The experiments described in the present study were carried out on keratinocytes (HaCaT) maintained in a complete culture *medium* (Dulbecco's Modified Eagle Medium enriched with 10% foetal calf serum). Cells were seeded in 96-well plates at a given concentration (1×10^4 cells/well) and were maintained under standard culture conditions (37°C, 95% RH, 5% CO_2) up to full confluence.

As menthyl nicotinate (whose commercial name is Nicomenthyl®) is lipophilic, it was first solubilized into maize oil and then added to the culture medium. Three menthyl nicotinate concentrations, 0.050, 0.025 and 0.013% were tested. Experimental conditions included both a negative control (CTR^-), that is, cells maintained in a culture *medium* supplemented with vehicles; and a positive control (CTR^+), that is, cells treated only using the chosen stress agent. The first condition corresponds to a basal, unstressed one; the second to the worst possible stress. The efficacy of the product has been tested considering these two conditions as a reference.

The chosen stress agents and their concentrations are hereafter described. Urban polluting agents were simulated with particulate matter and synthetic smoke. Particulate matter was PM10 loaded with a characterized and certified mixture of PAHs (polycyclic aromatic hydrocarbons), PCB (polychlorinated biphenyl), organochlorine pesticides; concentrations used were 0.25% for single exposure and 0.125% for repeated exposure. Synthetic smoke, used to replicate the insult on skin caused by cigarette smoke, was a 1:1:1:1 mixture of nicotine, cadmium, formaldehyde, ethyl carbamate; concentrations used were 0.00125% for single exposure and 0.0006% for repeated exposure. Hydrogen peroxide was chosen as a classic oxidizing agent (H_2O_2, 50 and 25 µM for single and repeated exposure respectively). The photo-ageing agent was the UV radiation emitted by a solar certified Suntest CPS^+ simulator; the delivered energy dose was 300 J/m^2 as a single exposure that was divided into three separate doses, given every 24 hours, for repeated exposure. Acute exposure involved a continuous 24 hours contact between the stress agents and the product. Repeated exposure was carried out, instead, for 72 consecutive hours (**Figure 2**).

Parameters being studied, and the corresponding biochemical methods, are hereafter reported. All tests were simple, standardized, accurate colorimetric assays. The protection indicator was calculated as the variation of each parameter in comparison with the positive control

Figure 2 – Positive control (CTR⁺): keratinocytes cultures exposed each to one of four stress agents (UV radiation, hydrogen peroxide, urban particulate matter, synthetic smoke), under acute (24 hours) and repeated (72 hours) exposure.

CTR⁺, exposed only to the stress agent.

Cell viability was evaluated using the MTT (3, (4,5-dimethylthiazol-2) 2,5 diphenyltetrazolium bromide) test (**4**).

This assay is based on the intracellular reduction of yellow tetrazolium salts by the mitochondrial succinate dehydrogenase into blue/purple formazan crystals. The reaction can occur only in cells that are metabolically active, and the corresponding optical density can be correlated with the number of viable cells. After being exposed to MTT, cells were incubated with isopropanol and the absorbance of the solutions was assessed using a microplate reader at 570 nm.

Cellular viability rate was calculated, for each experimental condition, as the ratio between the average optical density of exposed cell cultures and that of the negative controls.

The **protein metabolism** was evaluated through the protein synthesis assay devised by Lowry *et al*. (**5**). As in the biuret test, Cu^{2+} ions form complexes with proteins, under alkaline conditions, by catalysing the reduction of tyrosine and tryptophan residues. This oxidation causes the reduction of the Folin-Ciocalteu reagent that, from being initially distinctively yellow, turns blue; the more intense the blue, the more proteins are present in the biological matrix. Results are provided as the protein content (µL/mL) of the culture medium.

The **damage to the lipid component** is assessed by dosing the malondialdehyde (MDA), an index of lipid-specific oxidative stress. Lipoperoxides were quantified using the colorimetric assay developed by Erdelmeier and colleagues (**6**). This assay is based on the reaction between N-methyl-2-phenylindole (NMPI), a chromogen, and MDA, at 45°C and under acidic conditions, resulting in the production of a stable blue chromophore having a 586 nm absorption peak.

Quantitative determination follows by using a calibration curve created by measuring known, increasing concentration of an MDA standard. Results are expressed as the concentration of MDA (µM) in 100 µL of cell homogenate.

The **detox activity** was assessed by evaluating the activity of the glutathione S-transferase (GST), an enzyme belonging to a group of isoenzymes playing a pivotal role in detoxifying tissues. These enzymes protect cells from toxic compounds by attaching thiol groups of glutathione to these compounds, leading to their elimination.

Cells cultured under each experimental condition were homogenized and the detoxifying effect was assessed, on the homogenate, evaluating the activity of the enzyme in conjugating glutathione to 2,4-dinitrochlorobenzene (DNCB); this produces a stable complex showing a 340 nm absorption peak.

The GST activity is provided as nmol/mL/min for each cell homogenate.

Results and discussion

For the sake of brevity and ease of interpretation, the protective efficacy of the product against the different stress agents under consideration is presented in the form of the most significant and representative plots concerning each parameter under investigation.

Test results were statistically analysed through Student's t-tests. All variations in comparison to the positive control (CTR⁺) were statistically significant ($p<0.05$).

Responses to each stress condition were examined in detail. The assessment of the protective activity against UV radiation shows that the substance of interest preserves cell viability completely. Under single exposure to stress inducing conditions, viability is 100%, as in the basal, non-stressed condition. Under repeated exposure, viability is nearly 70% (**Figure 3**).

Concerning the oxidizing stress agent, the ingredient fully protects cells and significantly increases their metabolism in comparison to the basal levels of the negative control; under single and repeated exposure conditions the variations approximated 120% and 50%, respectively, in comparison to the positive control (**Figure 4**).

When cells were exposed to synthetic smoke, the antioxidizing activity assessment showed the ingredient was able to provide full protection and reduce basal lipoperoxide content in cell cultures. Variations exceeded 80%,

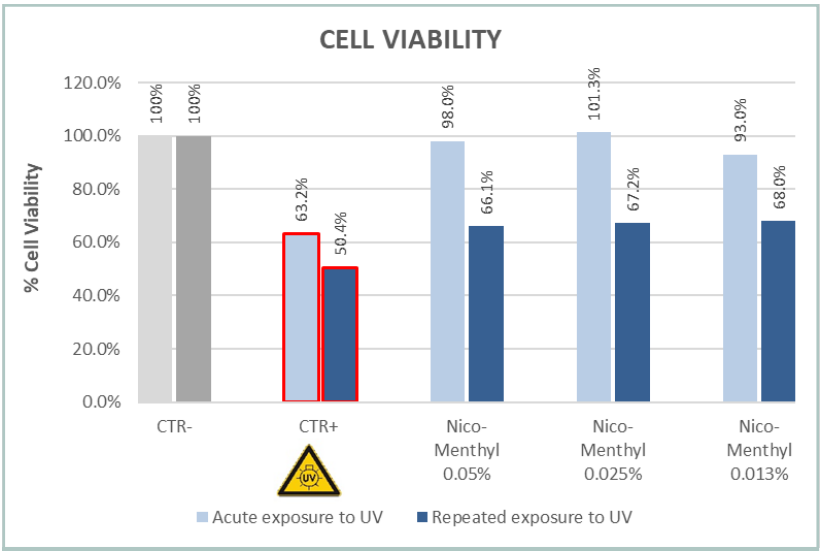

Figure 3 – Cell viability assessment after exposure to UV radiation and Nicomenthyl® treatment.

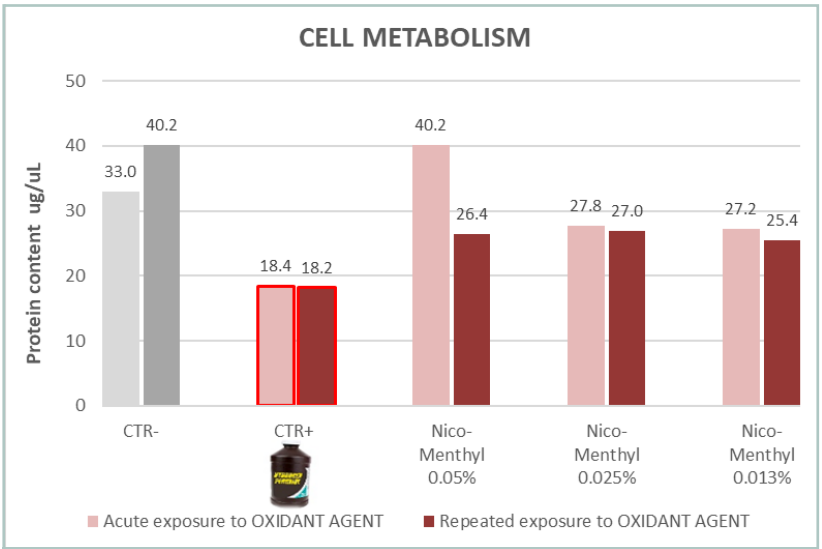

Figure 4 – Cell metabolism assessment after exposure to the oxidizing agent and Nicomenthyl® treatment.

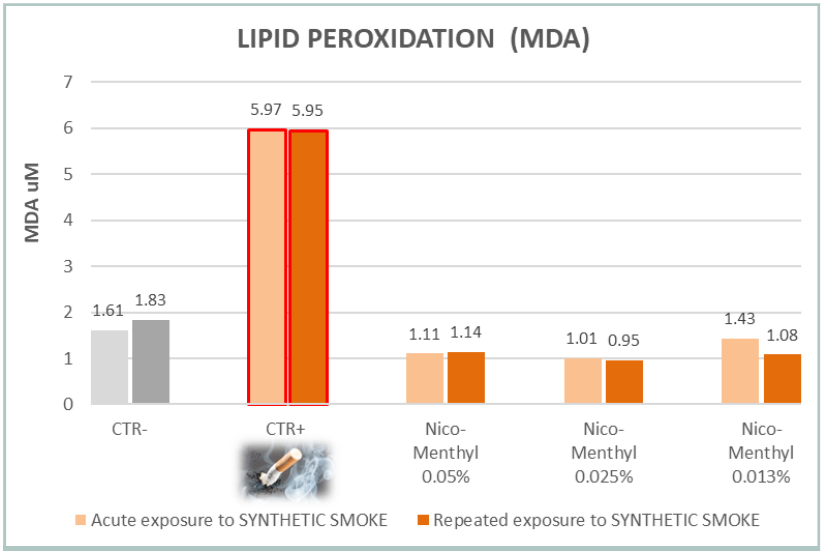

Figure 5 – Lipoperoxidation assessment after exposure to synthetic smoke and Nicomenthyl® treatment.

in both cases, in comparison to the positive control stressed with the damaging agent only (**Figure 5**).

The ingredient also shows a marked detoxifying effect, as observed assessing the enzymatic activity of the glutathione S-transferase (GST) when urban particulate matter was used as a stress agent. The substance brought increased GST activity, which had decreased because of the exposure to the stress agent, back to the basal value that could be observed in the negative control. Variations in comparison to the positive, stressed control exceeded 50% (**Figure 6**).

Summarizing (**Tables 2, 3 and 4**) results of the present study show that treating cell cultures with the stress agents caused significant variations in the biochemical parameters under assessment.

Conversely, exposing cells to the stress inducers together with the product of interest significantly mitigated the alterations caused by the stress agents, bringing back the parameters under consideration to their basal levels and, in some cases, improving them.

Conclusions

The results of this preliminary investigation show that the ingredient under examination may exert a significant protective action against the different damaging agents that have been considered, both in a single and a repeated exposure condition. Specifically, the *in vitro* system being used allowed to observe the ingredient has a significant antioxidizing, detoxifying, antipollution and metabolism-activating effect.

The ingredient fully penetrates the skin within 24 hours from application (experimental data on reconstructed epidermis, not shown in the present paper, which soon will be the subject of a separate publication). This is another positive characteristic that adds up to those presen-

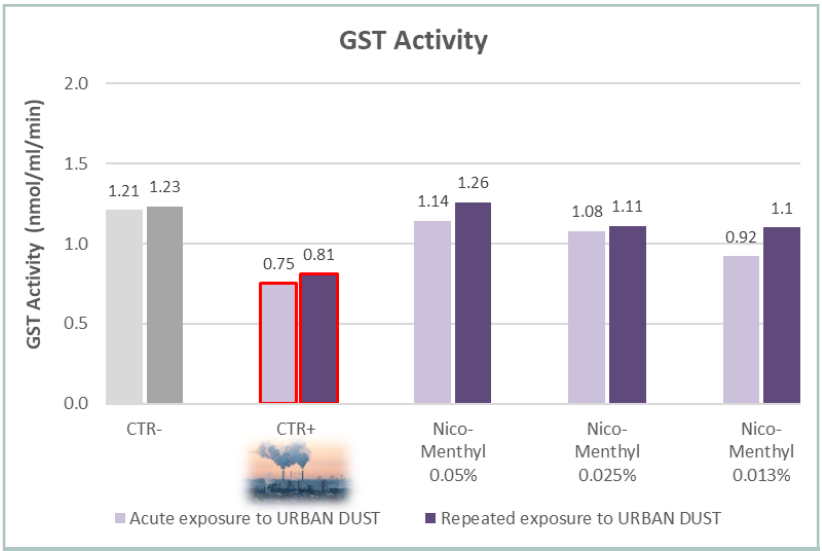

Figure 6 – GST activity assessment after exposure to urban particulate matter and Nicomenthyl® treatment.

Summary - variation (%) of biochemical parameters in cell cultures exposed to a damaging agent (CTR⁺) in comparison to basal values (CTR⁻)

Damaging agent	Test	Acute exp. 24h	Repeated exp.72h
UV radiation	Cell viability	↓ 63	↓ 50
Oxidizing agent	Cell metabolism	↓ 56	↓ 45
Synthetic smoke	Lipoperoxidation MDA	↑ 371	↑ 325
Urban PM	GST activity	↓ 62	↓ 66

Table 2 – Variation (%) of biochemical parameters monitored after exposure to each damaging agent.

Summary - Recovery (%) of biochemical parameters after Nicomenthyl® (NM) treatment in comparison to basal values (CTR⁻)

		NM 0.05%		NM 0.025%		NM 0.013%	
Damaging agent	Test	Ac. exp. 24h	Rep. exp. 72h	Ac. exp. 24h	Rep. exp. 72h	Ac. exp. 24h	Rep. exp. 72h
UV radiation	Cell viability	↑ 98	↑ 66	↑ ***101***	↑ 67	↑ 93	↑ 68
Oxidizing agent	Cell metabolism	↑ ***122***	↑ 66	↑ 84	↑ 67	↑ 82	↑ 63
Synthetic smoke	Lipoperoxid. MDA	↓ ***69***	↓ ***62***	↓ ***63***	↓ ***52***	↓ ***62***	↓ ***59***
Urban PM	GST activity	↑ 94	↑ ***102***	↑ 90	↑ 90	↑ 76	↑ 90

Table 3 - Recovery (%) of keratinocyte cultures treated with 0.05 – 0.025 – 0.013% Nicomenthyl® after exposure to each damaging agent, in comparison to basal values of negative control (CTR⁻). ***Bold italic*** entries mark observations representing an improvement compared to basal values.

Summary - Recovery (%) of biochemical parameters after Nicomenthyl® (NM) treatment in comparison to stressed positive control (CTR⁺)

		NM 0.05%		NM 0.025%		NM 0.013%	
Damaging agent	Test	Ac. exp. 24h	Rep. exp. 72h	Ac. exp. 24h	Rep. exp. 72h	Ac. exp. 24h	Rep. exp. 72h
UV radiation	Cell viability	↑ 55	↑ 31	↑ 60	↑ 33	↑ 47	↑ 35
Oxidizing agent	Cell metabolism	↑ 119	↑ 45	↑ 51	↑ 48	↑ 48	↑ 40
Synthetic smoke	Lipoperox. MDA	↓ 81	↓ 81	↓ 83	↓ 84	↓ 76	↓ 82
Urban PM	GST activity	↑ 52	↑ 56	↑ 44	↑ 37	↑ 23	↑ 36

Table 4 – Recovery (%) of keratinocyte cultures treated with 0.05 – 0.025 – 0.013% Nicomenthyl® after exposure to each stress agent, in comparison to values of stressed positive control (CTR⁺).

ted in this study, thus further supporting the efficacy of the ingredient that, passing the skin barrier, may easily reach the underlying anatomical site and there exert its action.

Its hydrolysis, and the consequent release of niacin, activates and supports the enzymatic reactions at the base of epithelial cell renewal, barrier function, immune system consolidation and DNA protection and repair (**3**).

Nicomenthyl® may therefore be regarded as valid formulative support to fight effectively all the negative effects our skin is increasingly exposed to today, caused by UV radiation, oxidizing agents, free radicals, urban particulate matter, smokes and toxins.

This ingredient has already been praised for its microcirculation activating properties, as a vehiculating agent for other lipophilic active ingredients and as a substance capable of providing a pleasant warm-cold sensation. Now the ingredient has been shown to be absolutely effective in all cosmetic formulas (such as sun protection and after-sun lotions for children and/or for delicate skin; or antipollution, anti-aging, anti-free radicals preparations for sensitive skin; or hair loss prevention formulations etc.) where the maximum protective, antioxidizing, antipollution, detox and reparative effect is sought.

Future study may provide in-depth insight into each single ingredient activity among those that have been the subject of this first investigation, considering other endpoints and using different experimental techniques to achieve a full understanding of the mechanism of action of this substance. In the future, clinical trials should also be carried out to expand the knowledge concerning this ingredient, to confirm the results of the present *in vitro* investigation and to further study its other promising functional and applicative characteristics.

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