A new skin protector and revitaliser from marine pine

FEDERICA D'ABROSCA

Res Pharma
In Vitro Toxicology and Applied Cellular Biology Laboratory
via G. Pastore, 3
Trezzo s/Adda (MI), 20056, Italy

ABSTRACT: The main role of oxidative stress of degeneration processes featured in skin ageing processes is widely recognised in literature (1-3). The damage caused, initially, is limited to skin cells only and then propagates in underlying tissues triggering a sub-clinical chronic inflammation that contributes to degeneration of skin's three-dimensional structure and to involvement of skin micro-circulation. Our study has proven that Pantrofina® PC is a multifunctional active substance able to protect skin cells (keratinocytes and fibroblasts) completely thanks to the synergy between its anti-oxidant and soothing activities. The studied product is an extract of marine pine bark (Pinus pinaster), the drug is constituted by a mix of polyphenolic derivatives (flavonoids, catechins, epicatechins, phenolic acids) whose biological activity is well known.

KEYWORDS: oxidative stress, antioxidant activity, soothing activity, Pinus Pinaster extract.

INTRODUCTION

Skin ageing is a process related to genetic factors and influenced by environmental factors. It may be defined as a gradual alteration of skin homeostasis and its functionality.
The two main processes involved in this type of skin modification are *chronological ageing* and *photoageing (4-6)*
The first is caused by the genetic heredity and only partially influenced by the environment. As age increases, renewal of the corneal layer is reduced, the skin barrier becomes less reactive, more permeable and exposed to exogenous substances, that is, the skin barrier integrity and its reaction capacity are impaired. As age increases, the energy metabolism of the cells loses efficiency and the subsequent energy deficit produces: a decrease in the synthesis of new macro-molecules (collagen, elastin, etc), a reduction in the replacement of existing and possibly damaged cells, a reduction of anti-oxidants mechanisms, enzymatic and otherwise (7, 8). Said degeneration process inevitably causes an accumulation of catabolites that favours triggering of a chronic sub-clinical reaction hardly ever perceived by the individual but responsible of several degeneration processes (9). Another consequence of skin ageing is a marked increase of Trans-epidermal Water Loss (TEWL), and a reduction of skin hydration and production of inter-cellular fats of the corneal layer. This type of ageing involves all of the body, but it is more evident on the most exposed skin (which becomes thinner, paler, and dryer) and on the hair.

Genetic characteristics and environmental and life style provoked stress are responsible for skin ageing

Photoageing overlaps to the other type of ageing in the areas most exposed to light (hands, face and neck) and shall be regarded as a degeneration process induced by the oxidative insult operated chiefly by the UV rays. Chronic exposure to sun radiation in fact induces formation of free radicals that cause damage to nucleic acids, proteins, fats, interfering with replication, differentiation and physiological cell turnover of tissue.
At a macroscopic dermatologic level free radicals are responsible of the appearance of blemishes and flaws of various types, like skin dyschromia, caused by accumulation of more or less marked creases. Lastly, it must not be forgotten that the free radicals induced by exposure to UV rays and not neutralised may cause pathological skin alterations like erythema, characterised by an increase of local blood flow, increase of permeability of vessels and subsequent formation of oedemas (10-13).
While chrono-ageing shall be regarded as an inevitable and basically unstoppable process, photoageing consequences can be reduced, by reducing its main triggering cause, that is formation of free radicals (UV), and by neutralising the free radicals produced.
Therefore, it is important to employ UV protection filters and antioxidants both in skin and hair preparations, as oxidative systems may have negative effects on hair as well.
Pinus pinaster is the botanical name of the plant, belonging to the *Pinaceae* family; it is commonly known as maritime or cluster pine.
The therapeutic effects of the extracts obtained from the bark of this plant have been well known for centuries: in Europe use of marine pine bark extract as a therapeutic substance dates back to 1967, in 1987 the extract has been registered as a brand, *Pycnogenol*, a brand which is still used. (14-17)
The *Pinus pinaster* bark extract has been discovered to contain a vast amount of polyphenolic derivatives, which are known to be the main reason for the strong anti-oxidant and anti-free radicals' effect of the extract (17-21).
The *procyanidines,* flavonoids with oligomeric structure (i.e. constituted by 2 to 7 flavonoid units bound together), are found in many plants (e.g. marine pine, grapes and apples) in which they work as antioxidants and protection from the attack of insects and micro-organisms. These molecules are certainly among the most important components of the drug: 1) they inhibit the enzymes that trigger the inflammatory

reaction thus limiting triggering of MMP metalloproteinasis that degrade the connective tissue (22-24); 2) they prevent formation of free radicals; 3) they reinforce the activity of anti-oxidant enzymes like superoxide dismutases (SOD) and glutathione peroxidases (Gpx); 4) they neutralise free radicals that were not reached by the cell metabolism.

In synergy with the antioxidant effect of procyanidines on the activity of xantino oxidasis the effect of phenolic acids is triggered: caffeic acid (acid 3.4-hydroxycinnamic) and pherulic acid (acid 4-hydroxy-3-methoxycinnamic) have proven to be efficient in neutralising free radicals of oxygen and nitrogen generated by photoexposure (25-28).

Pinus pinaster grows in extreme environmental conditions; it synthesizes and accumulates molecules in the bark which protect from external stress

In PPE (*Pinus pinaster* extract) we have also identified two isoflavonic glucosides, *daidzine* and *genistine*, which are biologically active in hydrolised form, as aglycones: *daidzein* and *genistein*. Genistein is a well know inhibitor of the protein tyrosine kinase and therefore it affects the metabolism of collagen of which it stimulates production. *(29-32)*

MATERIALS AND METHODS

Anti-radical activity

Cell model: a stabilised human keratinocytes line (HuKe) has been used.

This test is based on use of the 2',7'-dychlorofluorescein di-acetate (DCFH-DA) probe that penetrates into cells where it binds to intracellular microstructures and becomes fluorescent only after oxidation by the free radicals; measurement of the emitted fluorescence allows for a semi-quantitative assessment of the amount of free radicals generated (33, 34).

In order to confirm the anti-radical action of the extract the test has been carried out in parallel with two other known anti-radical agents like Vitamin C and solubilised Vitamin E acetate (internal controls).

Cells, treated with the selected PPE, Vitamin C and E doses (0.05 mg/ml) for 24 hours were then exposed to a 5μM DCFH-DA solution and then underwent an oxidative stress by means of an UVA lamp (SOL2 Hoenle): 4 subsequent 5J/cm^2 irradiations have been carried out and fluorescence has been evaluated with a FluoroCount^{TM} Packard fluorimeter (λ excitation 485nm, λ emission 530nm) after every irradiation and after one hour of "maintenance" after the last irradiation (20J/cm^2 +REC).

After completing the anti-radical activity test a cell vitality (Neutral Red Up-take) has been carried out to verify that the emitted fluorescence decrease was due to an actual abatement of free radicals and not to a cytotoxic and/or phototoxic effect on the tested sample on cells.

Soothing effect

Chrono-ageing cell model: a primary human fibroblasts line (Hude) and a stabilised line of human keratinocityes line (HaCaT) have been used.

Both cell lines, kept in culture for a limited time, face a natural ageing process that concludes with the release of pro-inflammatory mediators (Interleukins), said cell model has been regarded as similar to the physiological chrono-ageing process that happens *In Vivo, and therefore has been adopted.* In these conditions the soothing effects of PPE have been expressed through its capacity of inhibiting production and release of pro-inflammatory mediators Interleukins 8 and 6 in the culture medium (dosing of the two mediators has been carried out by applying the immunoenzymatic ELISA sandwich-type tests), the efficiency of the product has been evaluated by comparing it with the inhibiting activity of two well known soothing substances like Calendula (fluid extract 20°) and natural a-Bisabolol. The "aged" cells have been inoculated with selected doses of PPE, Calendula 2-1 and 0.5 mg/ml and a-Bisabolol 0.03 and 0.01 percent for a time of 24 hours, and after that time the IL6 and IL8 doses have been released in the culture medium.

Photoageing cell model: a primary human fibroblasts line (Hude) and a stabilised line of human keratinocityes line (HaCaT) have been used. The causes that generate photoageing are chiefly environmental and amongst them the most important is exposure to ultraviolet radiations (sunlight) and environmental pollution; For this reason the experimental photoageing module developed entails sensitising the cells with a $NiSO_4$ (1 mg/ml) solution and then exposing them with a dose of UVB of 0.024 mw/cm^2; in these conditions the cells produce high levels of IL8 and IL6 reproducing *In Vitro* the inflammation that occurs *In Vivo*. The soothing action has been obtained by exposing the previously sensitised cells to selected doses of PPE, Calendula and a-Bisabolol, and it basically consists of its ability of inhibiting production and release of pro-inflammatory Interleukin 8 and 6 in the culture medium (the dosing of the two mediators has been carried out by application of immunoenzymatic sandwich-type ELISA tests).

RESULTS

Anti-radicals activity

The results obtained after the first irradiation (5J/cm^2) testify that Pantrofina®PC has an excellent scavenger action comparable to Vitamins C and E (i.e. about 45 percent), however, in the subsequent irradiations only Pantrofina®PC retains a persistent level of inhibition of free radicals: one hour after the last irradiation (20J/cm^2) Pantrofina®PC still shows a 16 percent inhibition of free radicals formation while vitamins' activity, at this time, is depleted (Figure 1).

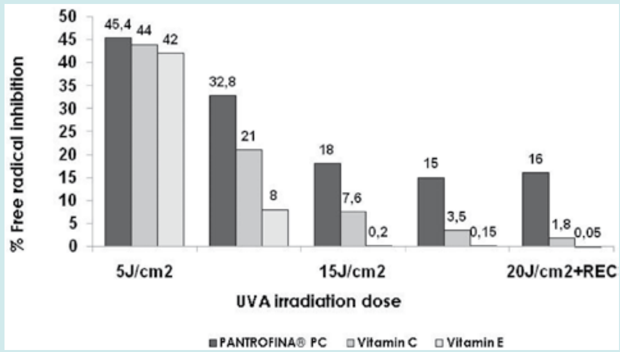

Figure 1. Antioxidant activity – dose 0,05 mg/ml.

Soothing effect

In physiological conditions (no sensitisation), Pantrofina®PC and Calendula have proven to have substantially the same efficiency (Figure 2) on inhibition of release of IL8 by keratinocytes, however on the sensitised cells PPE has proven to be much more efficient than Calendula in inhibiting release of IL8 and IL6 (Figures 3, 4).

The results obtained by comparing soothing action of PPE and a-Bisabolol in physiological conditions (Figure 5), have pointed out significant differences between the two active substances about the release of IL8, while only PPE has proven to be efficient in release of IL6 (Figure 6).

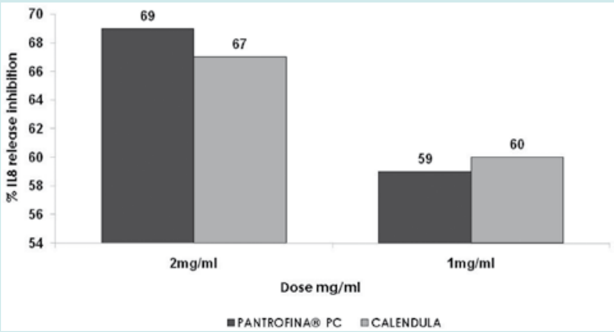

Figure 2. Soothing activity – non sensitized keratinocytes.

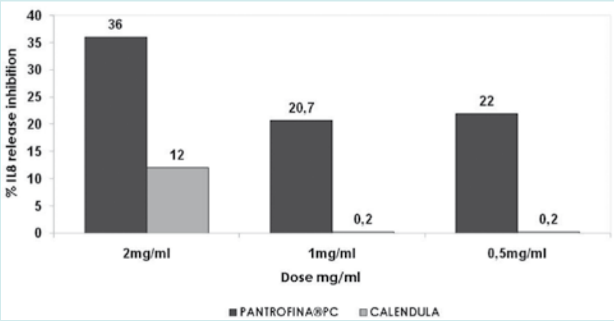

Figure 3. Soothing activity – keratinocytes sensitized with NiSO4.

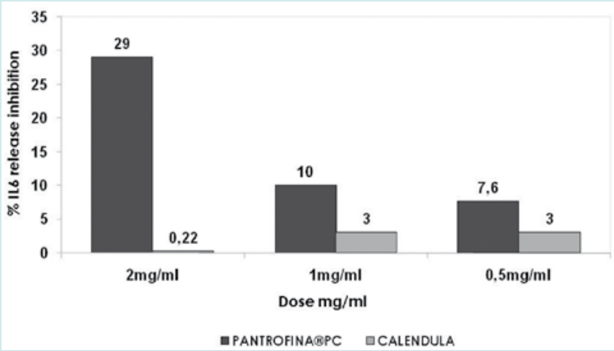

Figure 4. Soothing activity – keratinocytes sensitized with UVB.

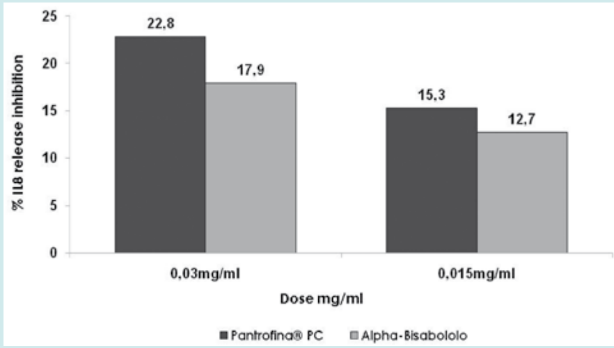

Figure 5. IL8 release inhibition – non sensitized fibroblast.

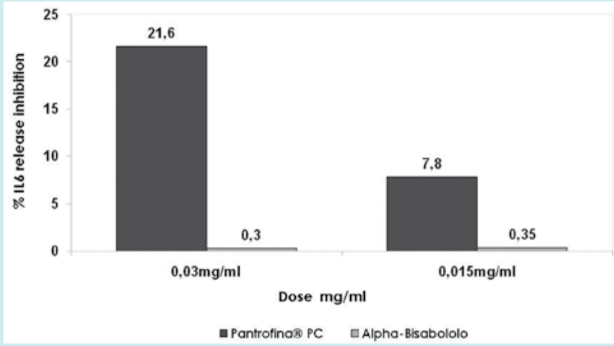

Figure 6. IL6 release inhibition – non sensitized fibroblast.

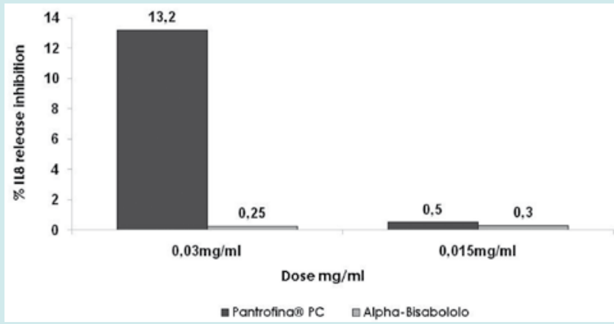

Figure 7. IL8 release inhibition –fibroblast sensitized with UVB.

As regards the experimental model of photoageing the results obtained pointed out that only PPE is capable of significantly inhibiting IL8 release by fibroblasts (Figure 7), while neither has been able to reduce the release of IL6 (unpublished results).

PPE and activation of cell vitality

In order to demonstrate PPE's regenerating-protection action, we have carried out a morphological analysis on the cells (HaCaT) exposed to stress by irradiation of UVB (0.024 mw/cm^2) and then left to recover for one night (22-24 hours) in presence of the active substance (0.01 percent). At the end of the incubation period the PPE effect has been evaluated by comparison with the positive control group represented by cells exposed to UVB radiations and stored for the next night with the culture medium alone (morphological observation with a phase contrast microscope -Nikon ECLIPSE TS 100- and acquisition of digital images). Analysis of images clearly shows that PPE can reduce the damages caused by UVB irradiation at both doses of 0.005 and 0.125

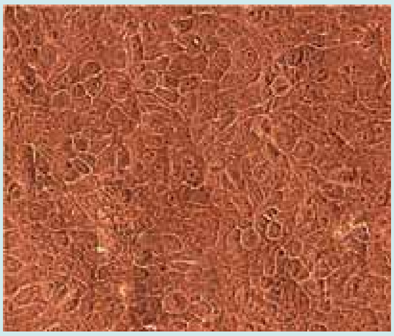
Image 1. Negative control.


Image 2. Positive control.

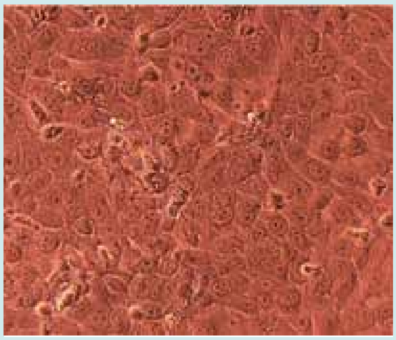
Image 3. Keratinocytes irradiated with UVB and then treated with PANTROFINA PC 0.005 mg/ml.

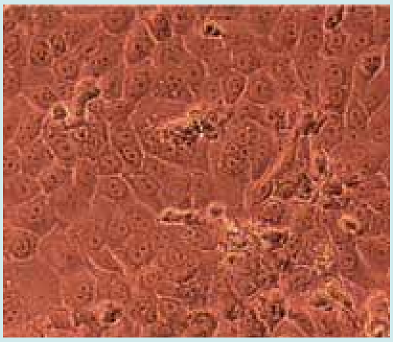

Image 4. Keratinocytes irradiated with UVB and then treated with PANTROFINA PC 0.0125 mg/ml.

mg/ml (Image 3 and 4), as here the dead cells (black dots) and the suffering cells, undergoing detachment are fewer with respect to the positive control group (Image 2). Image 1 shows not irradiated and not treated cells (negative control). These results confirm and summarise the results obtained by the above described tests and prove PPE's effectiveness in protecting against oxidative stress and the subsequent inflammation triggered: the active principle action produces an improvement of cell vitality in extreme culture conditions.
In other words, PPE grants to cells a physiological support when they need them most: during chrono- and photo-ageing.

DISCUSSION

Analysis of obtained results outlines a multi-functional active substance that works at several levels on different metabolic processes causing a final functional effect reinforced by the synergy of anti-oxidant and anti-inflammatory actions.
The obtained results show that the anti-radicals effect of Pantrofina®PC is probably due both to neutralisation of induced free radicals and a more complex action of reinforcement of the anti-oxidation (enzymatic or otherwise) defences eukariotic cells are inherently equipped with. The free radicals formation process of cells exposed to Pantrofina®PC and then irradiated by UVA rays is more gradual over time, a fact which could hardly be explained simply by the drug's being able to "restrain" free radicals: if that was the case, the "biochemical" consumption of the anti-oxidant activity would lead to a more drastic interruption of the scavenger activity, which for example happens in the results obtained with vitamins C and E.
From these results it is evident that PPE's soothing effect is stronger than a-Bisabolol's and Calendula's effects, which can be presumably ascribed to the synergy between the substance's anti-oxidant and soothing effects. This active substance, in fact, in addition to having a purely soothing action, neutralises formation of free radicals, thus restraining the inflammation caused by UVB irradiation, while a-Bisabolol, conversely, has simply a soothing effect and consequently a narrower field of action.
Judging from the above, it is easy to infer the likely fields of usage for PPE (marketed by Res Pharma srl, Milan, with the registered trademark *Pantrofina PC') are*:

- anti-ageing (anti-wrinkle) creams, recommended also for lotions and shock therapy vials with higher product concentration and therefore a stronger activity
- sun protection products (before/after suntan)
- soothing products, shaving products, aftershave, after depilation
- soothing products in general, for face, hands and body treatment.

The extract carrier
PPE consists of an extract of *Pinus Pinaster* bark, solubilised in diglycerin, a humectant agent with less hydroxyl groups than

other hydrating agents commonly used in cosmetics (glycerine, propylene glycol and sorbitol in particular). It therefore features a reduced capacity of absorbing water, but the absorbed water is released more slowly, therefore the products using DG as a carrier have a longer lasting humectant capacity and consequently more significant soothing and lubrication effects but with reduced stickiness. Diglycerin (DG) therefore confers an added value to an already very effective active substance.

Marine pine extract contrasts skin ageing thanks to the synergy between it's antioxidant and soothing activities

REFERENCES AND NOTES

1. M. Zhu, G. Tim Bowden, "Molecular Mechanism(s) for UV-B Irradiation-Induced Glutathione Depletion in Cultured Human Keratinocytes", *Photochemistry and Photobiology,* **80**, pp. 191-196 (2004).
2. K. Tanaka et al., "Prevention of the Ultraviolet B-Mediated Skin Photoaging by a Nuclear Factor kb Inhibitor, Parthenolide", *The Journal of Pharmacology and Experimental Therapeutics*, **315**, pp. 624-630 (2005).
3. R. David Bickers, M. Athar, "Oxidative Stress in the Pathogenesis of Skin Disease", *Journal of Investigative Dermatology*, **126**, 2565-2575 (2006).
4. A. Slominski, J. Pawelek, "Animals under the Sun: Effects of Ultraviolet Radiaton on Mammalian Skin", *Clinics in Dermatology*, **16**, pp. 503-515 (1998).
5. S. Lisby et al., "UV-induced DNA damage in human keratinocytes: Quantitation and Correlation with long-term survival", *Experimental Dermatology*, **14**, pp. 349-355 (2005).
6. L. Naderi-Hachtroudi et al., "Induction of Manganese Superoxide Dismutase in Human Dermal Fibroblasts", *Arch Dermatol.*, **138**, pp. 1473-1479 (2002).
7. S. Basu-Modak et al., "Epicatechin and its methylated metabolite attenuate UVA-induced oxidative damage to human skin fibroblasts", *Free Radicals Biology & Medicine,* **35(8)**, pp. 910-921 (2003).
8. B. Cestaro, "Per una vita inossidabile", *Apporti nutrizionali del latte e derivati*, Etaslibri (RCS Medicina) (1990).
9. M.S. Duthie et al., "The effects of ultraviolet radiation on the human immune system", *British Journal of Dermatology*, **140**, pp. 995-1009 (1999).
10. A. Pelosi, E. Berardesca, "Aspetti dermatologici dell'invecchiamento cutaneo", *Kosmetica,* **5**, pp. 55-59 (2001).
11. P.C. Pantrofina, Documentazione Centro Tecnico Res Pharma, Trezzo s/Adda, Mi (2009).
12. C.D. Enk et al., "Photoprotection by Cichorium endivia Extracts: Prevention of UVB-Induced Erythema, Pyrimidine Dimer Formation and IL-6 Expression", *Skin Pharmacol Physiol*, **17**, pp. 42-48 (2004).
13. P.A. Petland et al., "Enhanced Prostaglandin Synthesis after Ultraviolet Injury is Mediated by Endogenous Histamine Stimulation", *-J. Clin. Invest.*, **86**, pp. 566-574 (1990).
14. G. Bregaglio, L. Erba et al., "Pinus pinaster: antiossidante ed antiradicalico nell'ageing", *Cosmet & Technol.*, **5(4)**, pp. 29-35 (2002).
15. B. Rihn, C. Saliou et al., "From ancient remedies to modern therapeutics: Pine bark uses in skin disorders revisited", *Phytother Res,* **125**, pp. 76-78 (2001).
16. F. Chandler, L. Freeman et al., "Herbal remedies of the maritime indians", *J Ethnopharmacol.*, **1**, pp. 49-68 (1979).
17. L. Packer, G. Rimbach et al., "Antioxidant activity and biological properties of a procyanidin-rich-extract from Pine (Pinus maritima) bark", *Free Radical Biol Med.*, **27(5, 6)**, pp. 704-724 (1999).
18. P. Rohdewald, "A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology", *Int J Clin Pharmacol Ther.*, **40(4)**, pp. 158-168 (2002).
19. A.L. Molan et al., "Antioxidant activity and poliphenol content of green tea flavan-3-ols and oligomeric proanthocyanidins", *Int J Food Sci Nutr.*, **12**, pp. 1-10 (2008).
20. Y. Kimura et al., "Characterization and Antioxidative Properties of Oligomeric Proanthocyanidin from Prunes, Dried Fruit of Prunus domestica L.", *Biosci. Biotechnol. Biochem.*, **72(6)**, pp. 1615-1618 (2008).
21. S. Kayano et al., "A New Bipyrrole and Some Phenolic Constituents in Prunes (Prunus Domestica L.) and Their Oxygen Radical Absorbance Capacity (ORAC)", *Biosci Biotechnol Biochem.*, **68(4)**, pp. 942-944 (2004).
22. A. Schafer et al., "Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol)", *Biomed Pharmacother*, **60(1)**, pp. 5-9 (2006).
23. K.J. Cho et al., "Inhibition mechanisms of bioflavonoids extracted from the bark of Pinus maritima on the expression of proinflammatory cytokines", *Ann N Y Acad Sci.*, **928**, pp. 141-156 (2001).
24. A.K. Greul et al., "Photoprotection of UV-irradiated human skin: an antioxidative combination of vitamins E and C, caotenoids, selenium and proanthocyanidins", *Skin Pharmacol Appl Skin Physiol.,* **15(5)**, pp. 307-315 (2002).
25. O.Y. Lin *et al*., "Inhibition of inducible nitric oxide synthase by Acanthopanax senticosus extract in RAW264.7 macrophages", *J Ethnopharmacol.*, **118(2)**, pp. 231-236 (2008).
26. X. Terra et al., "Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NfkB signaling pathway", *J Agric Food Chem.*, **55(11)**, pp. 4357-4365 (2007).
27. C. De Sigis, "Pycnogenolo ® in Cosmetica", *Fitocosmesi*, pp. 34-38.
28. C.G.M. Heijnen et al., "Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups", *Toxicology in Vitro*, **15**, pp. 3-6 (2001).
29. V. Schultz, R. Hansel et al., *Rational phytotherapy: a physician guide to herbal medicine*, Springel, Berlin (1997).
30. E. Bombardelli, P. Morazzoni, "Vitis vinifera", *Fitoterapia,* **66**, pp. 291-317 (1995).
31. A. Saija, A. Tomaino et al., *Sci Food Agric.*, **3**, pp. 476-480 (1999).
32. D. Schimdt, C. Hanay et al., "Genistein, a new cosmetic ingredient derived from soy", SÖFW J., **127(10)**, pp. 22-27 (2001).
33. G. Anumantha et al., "Reactive oxygen species by cyanide mediate toxicity in rat pheochromocytoma cells", *Toxicology Letters*, **93**, pp. 47-54 (1997).
34. P.C. Lepel et al., "Organometal-Induced increases in oxygen reactive species: the potential of 2',7'-Dichlorofluorescin diacetate as an index of neurotoxic damage", *Toxicology and Applied Pharmacology*, **104**, pp. 17-24 (1990).