SEA HEATHER®

The best radical scavenger from the Mediterranean

*

Combats damage induced by the
both pathways of the lipid peroxidation

Protects DNA

Soothes irritated skin

Gelyma

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SUMMARY

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INTRODUCTION

Oxygen sustains aerobic life on our "Blue Planet". It is fundamentally essential for energy metabolism and respiration but it is also implicated in many diseases and degenerative conditions (Marx, 1985 – Science 235: 529-531).

Approximately, 3.5 kilograms of oxygen are consumed per head. About 2.8% is not properly used and form free reactive oxygen species.

In its ground-state, atmospheric oxygen is a biradical. It bears two impaired electrons. It is usually non-reactive to organic molecules which have paired electrons with opposite spins.

To participate in reactions with organic molecules, oxygen may be "activated".

This activation occurs according to two different mechanisms which result in either a singlet state or a triplet state.

The singlet oxygen is not a true radical but it is an extremely reactive form. It is reported to be an important oxygen species in reactions related to ultraviolet exposition. It is able to rapidly oxidizing many molecules such as amino-acids, enzymes and lipids.

From its triplet state, oxygen can be activated by reduction. It generates several oxygen reactive species. The first reduction forms superoxide. Subsequent reductions form hydrogen peroxide, hydroxyl radical and water.

All these reactive oxygen species cause important cellular damage. They can degrade cellular DNA, oxidize proteins and alter membrane lipids (Fridovich & Porter, 1981 – J. Biol. Chem. 256: 260-265; Brown & Fridovich, 1981 – Arch. Biochem. Biophys. 206: 414-420).

The mechanisms by which oxygen radicals damage membranes are associated with peroxidation reactions in membrane lipids.

The complexity of biological membranes is well established. The lipid bilayer membrane is composed of a mixture of phospholipids and glycolipids that have fatty acid chains attached to carbons 1 and 2 of the glycerol backbone by an ester linkage.

The peroxidation reactions differ among these fatty acids depending on the number and position of the double bonds on the acyl chain (Frankel, 1985 – Progress in Lipid Research 23: 197-221).

Thereby, lipoperoxidation is particularly destructive. It occurs according to two different mechanisms: a non-enzymatic system and an enzymatic system. (Emerit & Galli, 1987 – Cah. Nutri. Diét. 22: 35-.39).

In the former system, the unsaturated fatty acids are not selectively degraded. All membranes may be attacked, especially mitochondrial membranes by modification of energetic flux and lysosomial membranes by release of hydrolases. This mechanism known as the autoxidative peroxidation implies chain reactions (see page 11).

In the later system, a selective degradation concerns the arachidonic acid and implies the action of enzymes such as phospholipases. The arachidonic cascade is induces and generates inflammation (p.14). The arachidonic acid is converted into mediators of inflammatory processes, through the activity either of 5-lipoxygenase or the cyclooxygenase.

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Owing to these two mechanisms, lipoperoxidation induce numerous lesions in biological systems.

Consequently, it appears important to maximize skin protection against the lipid peroxidation on account of its implication in membrane damage and inflammatory processes.

In view of these considerations, GELYMA proposes

SEA HEATHER®

a global anti-peroxidative defence system

highly efficient

- to bust up the attacks of reactive oxygen species
- to fight inflammation.

It is derived from Mediterranean caespitose brown algae.

GELYMA Patent FR 2 838 341.

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THE BROWN ALGAE CAESPITOSE *Cystoseira*

The genus *Cystoseira* was established by C.A. Agardh in 1820. It belongs to the phylum *Heterokontophyta*, the class *Phaeophyceae*, the order Fucales and the family *Cystoseiraceae.*

The most comprehensible accounts of the morphology and biology of these brown algae are those of Sauvageau (1912 – Bull.Stn biol. Arcachon 14: 1-424), Ercegovic (1952 – Fauna Flora Adriatica 2 :1-212) and Roberts (1967- Br. Phycol. Bull. 3: 345-366).

Phycologists consider that the genus *Cystoseira* is still imperfectly known. So difficulties are inevitable in selection of suitable criteria for species speciation in field recognition (*cf.* Roberts – 1978 – In Modern Approaches to the taxonomy of red and brown algae, pp. 399-422, Academic Press).

Generally, specific separation is based on various criteria *e.g*. details of morphology, features of reproductive morphology and ecological criteria.

➤ Morphology & Biology

The *Cystoseira* species chosen for SEA HEATHER® belong to the group of algae described by Sauvageau (1912 – *Ibid*) as showing a caespitose basal part given rise to numerous upper axes.

It includes different species known as *C. caespitosa*, *C. amentacea* variety *stricta*, *C. brachycarpa* variety *balearica* (= *C. balearica*).

The figures 1 to 4 show morphological aspects of these algae which differ from each other only in small points of detail such as the morphology of the apical region of the main axis and the position of reproductive structures along axes.

The thallus of caespitose *Cystoseira* can reach up to 30-45 cm in height.

The basal part is large, caespitose and fixed to rocky substratum. It is perennial that allows renewing the upper system.

The branched system appears highly differentiated with numerous cylindrical branches.

The main axis is erect and divided into several branches, each pinnately subdivided to varying degrees.

Branches bear numerous spine-like appendages, cryptostomata and small dilations known as “air-vesicles” or “aerocysts”.

Spine-like appendages are known as “leaves” in many accounts.

Fig.1 – Morphology of juvenile iridescent submerged axes of *Cystoseira amentacea* variety *stricta.* Photo GELYMA.

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The apex of the main axis shows small morphological differences between the three species.

In *C. caespitosa* the main axis is flattened whereas in *C. amentacea* variety *stricta* and *C. brachycarpa* variety *balearica*, it is scarcely protruding. Other morphological criteria refer to the presence of absence of spine-like appendages around this apical region.

The young axes of *C. amentacea* variety *stricta* (Fig.2) and *C. brachycarpa* variety *balearica* are iridescent when submerged whereas *C. caespitosa* does not present any iridescence.

Fig.2 – Morphology of *Cystoseira amentacea* variety *stricta* in summer. Photo GELYMA.

The vegetative cycle is similar for the three species.

Growth is by means a single, 3-sided apical cell.
It is very active in winter and spring. In summer it reduces. In autumn, all upper branched system becomes decideous and leaves scars or stumps of varying degrees of prominence

The receptacular regions (reproductive structures) vary in appearance according to whether the conceptacles are in the basal parts of spine-like appendages or immersed in the axis itself.

Conceptacles are hermaphrodite. They include numerous tufted antheridia and one ovoid oogonia which occupies the basal region.

In *C. amentacea* variety *stricta* and *C. brachycarpa* variety *balearica*, conceptacles are grouped into receptacles produced seasonally. Receptacles develop from the ultimate ramuli present in the richly branched regions. They are oblong-cylindrical and clothed with spirally arranged “leaves”.

In *C. caespitosa*, conceptacles are non-grouped into receptacles.

For active manufacturing, only the long main axes of these algae (which naturally come off at the end of the vegetative period) are collected. The basal part, firmly attached to rocky substratum, is never cut off in order to renew the upper system and to preserve populations

Fig.3 – Morphology of *Cystoseira brachycarpa* variety *balearica* in summer. Photo GELYMA.

Fig.4 – Morphology of *Cystoseira caespitosa* in summer. Photo GELYMA.

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➤ Ecology & Geographical distribution

The genus *Cystoseira* C. Agardh 1820 is a genus of worldwide distribution with about 80% of the species occurring along the Mediterranean and adjoining Atlantic coasts.

According to the views of Feldmann (1937 – Rev. algol. 10: 1-339), Ercegovic (1959 – Int. Rev. Ges. Hydrobiol. Hydrogr.44:473-483), Giaccone (1971 – Natura e Montagna 4 : 41-47; 1974 – Mem. Biol. Mar. Oceanogr. 4: 149-168) and Giaccone & Bruni (1971 – Ann. Univ. Ferrara, N.S., Sezione IV, Botanica 4:45-70), many of nearly 30 Mediterranean species are neoendemic.

In the Mediterranean, *Cystoseira* species play the role of canopy algae taken over in cold temperate regions by the Laminariales. Their biomass reaches there 5kg.m^{-2}.

The remaining species of *Cystoseira* occur off the European North Atlantic coasts, the Pacific coasts of North America, the Sea of Okhotsk, Indian subcontinent, the Red Sea and Persian Gulf, the Sea of Japan and in Indonesia and southern and eastern Australia.

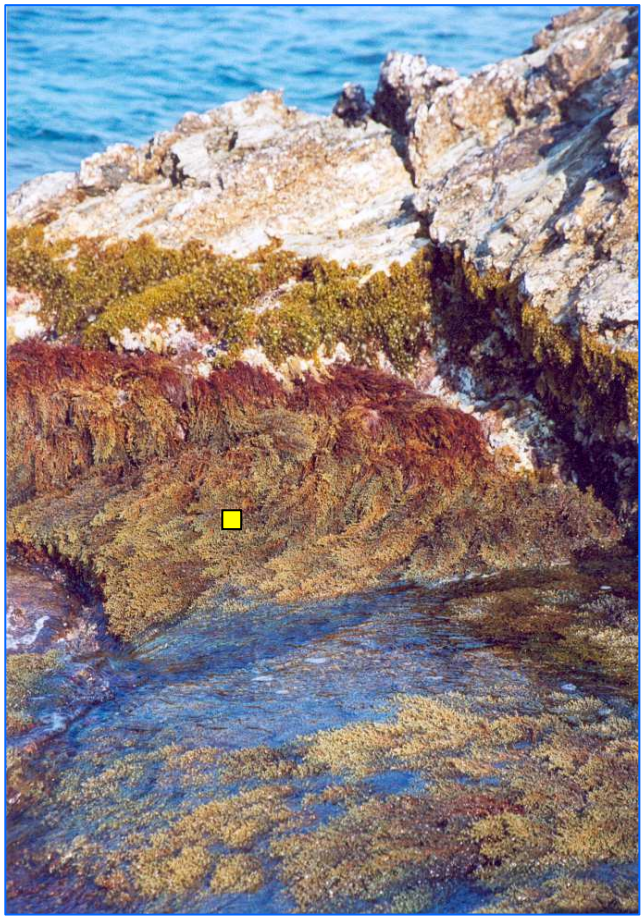

Fig. 5 - Aspect of the population of *Cystoseira amentacea* var. *stricta* ( ) in summer along the coasts of eastern Mediterranean. Photo GELYMA.

Caespitose *Cystoseira* species only occurs along the Mediterranean coasts, especially in the Western part, at the Côte d'Azur and in Algeria.

They constitute dense populations grouped into large belts generally at wave-exposed places (Fig. 5). They do not tolerate prolonged emersions. They cannot grow in polluted areas.

It is possible that some species, currently included in the genus *Cystoseira*, evolve into new entities, the genus *Cystoseira* being probably still in a process of active speciation (Luning, 1990 – Seaweeds, their environment, biogeography and ecophysiology, Wiley).

➤ Chemical composition and Utilizations

The chemical composition of *Cystoseira* species was studied by Pellegrini & Pellegrini (1971- Bot. mar. 14:6-16; 1972- Soc.Phycol.Fr Bull, 17:46-61).

As the other brown algae, they contain minerals, mannitol, laminaran and alginic acid. Seasonal variations in their chemical composition are present.

None industrial utilization is known for these seaweeds.

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THE ACTIVE INGREDIENT SEA HEATHER®

Specifications

on a control batch

- appearance : limpid liquid brown coloured
- odour : typical
- pH : 5.0 ± 1.
- density : 1.015 ± 0.010
- dry residue (%) : 2.7 ± 0.4
- solubility : soluble in ethanol, propylene glycol, butylene glycol
  : insoluble in oils
- microbiology : bacteria : < 100 germs / ml.
  : yeasts, moulds : < 100 germs / ml.
  : pathogens : free.

Composition

Ingredients		Amounts %
Solvent	water	52
Brown alga	*Cystoseira amentacea / caespitosa brachycarpa* extract	48
Preservative	*as required*	
Others (antioxidants …)	none	

INCI names water CAS n° 7232-18-5 EINECS n° 231-791-2
Cystoseira amentacea / caespitosa / brachycarpa extract

Storage

SEA HEATHER® should be stored in the original sealed drums, under clean conditions between 15 to 25°C. In order to avoid microbial secondary contamination, it is recommended to use the whole content of the drum once opened.

If stored under the recommended conditions, SEA HEATHER® remains stable for at least 18 months.

Pack size: 1kg - 5kg -10 kg.

Safety

No animal experimentation.

Standard safety testing proves that SEA HEATHER® is safe for cosmetic use.

SEA HEATHER® exhibits a slightly irritant potential for ocular irritation and a non irritant potential for dermal irritation at the recommended use levels.

No direct genotoxic effect is detected (3D assay).

cf. Annex pp. 21-23.

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EFFECTIVENESS EVALUATION

SEA HEATHER®

Nucleic acids

Protection of DNA → Protection against singlet oxygen

Membrane lipids

LIPID PEROXIDATION
Nonenzymatic pathway

Protection against cellular lysis →
- Protection against superoxide anion & hydrogen peroxide
- Protection against alkoxyl radical

LIPID PEROXIDATION
Enzymatic pathway

Protection against inflammation →
- Anti-phospholipase A2 activity
- Anti 5-lipoxygenase activity

Anti irritation properties →
- Cytological studies on reconstituted human skins
- Reduction of stinging sensations *In vivo* study

↓

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Protection of DNA

Protection against singlet oxygen

Singlet oxygen is formed from direct action to light on the oxygen molecule. It is not a true radical but it is reported to be an important reactive oxygen species in relation to ultraviolet exposition.

It diffuses easily and becomes very reactive, especially on DNA in which it induces damage such as single and double strand chain breaks, triggering lethal and mutagenic effects. Its toxicity is reinforced when appropriate photoexcitable compounds known as sensitizers (such as flavins, hemoproteins and reduced pyridine nucleotides) are present with molecular oxygen.

It has been also suggested that singlet oxygen may be formed during the degradation of lipids peroxides and thus may cause the production of other peroxide molecules.

Damaged Detection DNA (3D) assay

Method

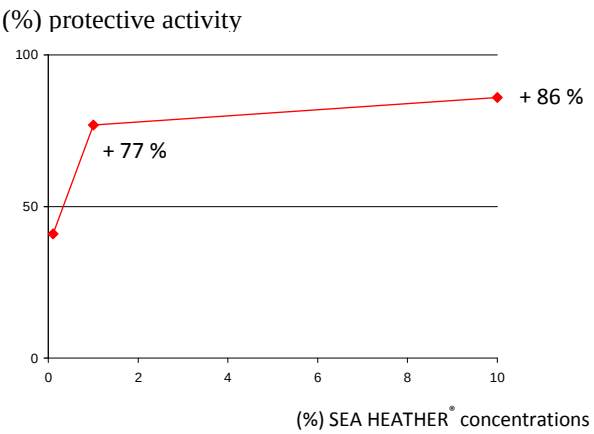
The chemiluminescent 3D Assay was performed by the well known company S.F.R.I. (St Jean d'Illac, France). It uses plasmid DNA adsorbed on sensitized microplates. It is based on a repair reaction of DNA (Salles *et al.*, 1995 – Analytical Biochemistry 232: 37-42, Patent FR n° 95003230).
DNA lesions are repaired by the excision repair pathway which implies an incision-excision reaction followed by DNA repair synthesis.
In the present experiment, these lesions were induced by the generation of singlet oxygen performed by photoactivation of methylene blue. Three concentrations of SEA HEATHER® were tested: 0.1 – 1 and 10%. The chosen standard was silymarine applied according to 3 concentrations: 1 – 10^{-1} and 10^{-2} mg.ml^{-1}.

Results

Compounds	Concentrations	% protection in presence of reactive oxygen species	% non specific inhibition	% specific protection	Concentration giving 50% of protection
SEA HEATHER®	10% 1% 0.1%	90 81 53	4 4 12	86 77 41	0.2%
Silymarine	1 mg.ml^{-1}. 10^{-1} mg.ml^{-1}. 10^{-2} mg.ml^{-1}.	79 68 11	5 7 0	74 61 11	0.06 mg/ml

➤ SEA HEATHER® shows a dose-dependent protection of DNA against damage caused by singlet oxygen.

IC 50 is reached with only 0.2% SEA HEATHER®.



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Protection against cellular lysis

The polyunsaturated fatty acids may be oxidized by both autoxidative and enzymatic peroxidation in free radical chain reactions (Hsieh & Kinsella, 1986 – J.Food Sci., 51:940-945; Eving *& al*., 1989 – Lipids 24:609-615).

In the enzymatic system, the arachidonic acid cascade is induced.

In the non enzymatic system, all membranes are attacked following the autoxidation of unsaturated fatty acids, especially mitochondrial membranes with changes in energy flux and lysosomal membranes with hydrolase release. Other consequences are altered fluidity, increased permeability and deactivation of cellular enzymes and transporters (*cf* reviews Pryor, 1978 – Photochemm.Photobiol. 28:787-801; Girotti, 1985 – J. Free Rad. Biol. Med. 1:87-95; Black, 1987 – Photochem. Photobiol. 46:213-221).

The survival of a cell depends on the integrity of its membranes.

Membranes must be fluid; that is why membrane lipids must be protected from oxidation.

Oxidized fatty acid chains are more hydrophilic than non-oxidized ones. They seek to migrate to the surface of the membrane to interact with water. This can disrupt the membrane structure.

Unfortunately, polyunsaturated fatty acids are easily attacked by reactive oxygen species, starting a chain-reaction known as the autoxidative system of lipoperoxidation.

This chain reaction includes different successive steps as illustrated page 10.

An alkyl radical L° is formed from lipid LH at the initiation step.

Then, the alkyl radical reacts with oxygen to constitute a lipid peroxylalkyl radical LOO° which reacts with an additional lipid molecule to produce a lipid hydroperoxide LOOH and a new alkyl radical L° at the propagation step.

In presence to metals, the hydroperoxide gives an alkoxyl radical LO° which associated with a lipid gives a non radical compound and an alkyl radical (reactivation step). This reactivation step may restart the chain reaction.

At the termination step, lipid peroxyl radicals may combine to produce non radical compounds.

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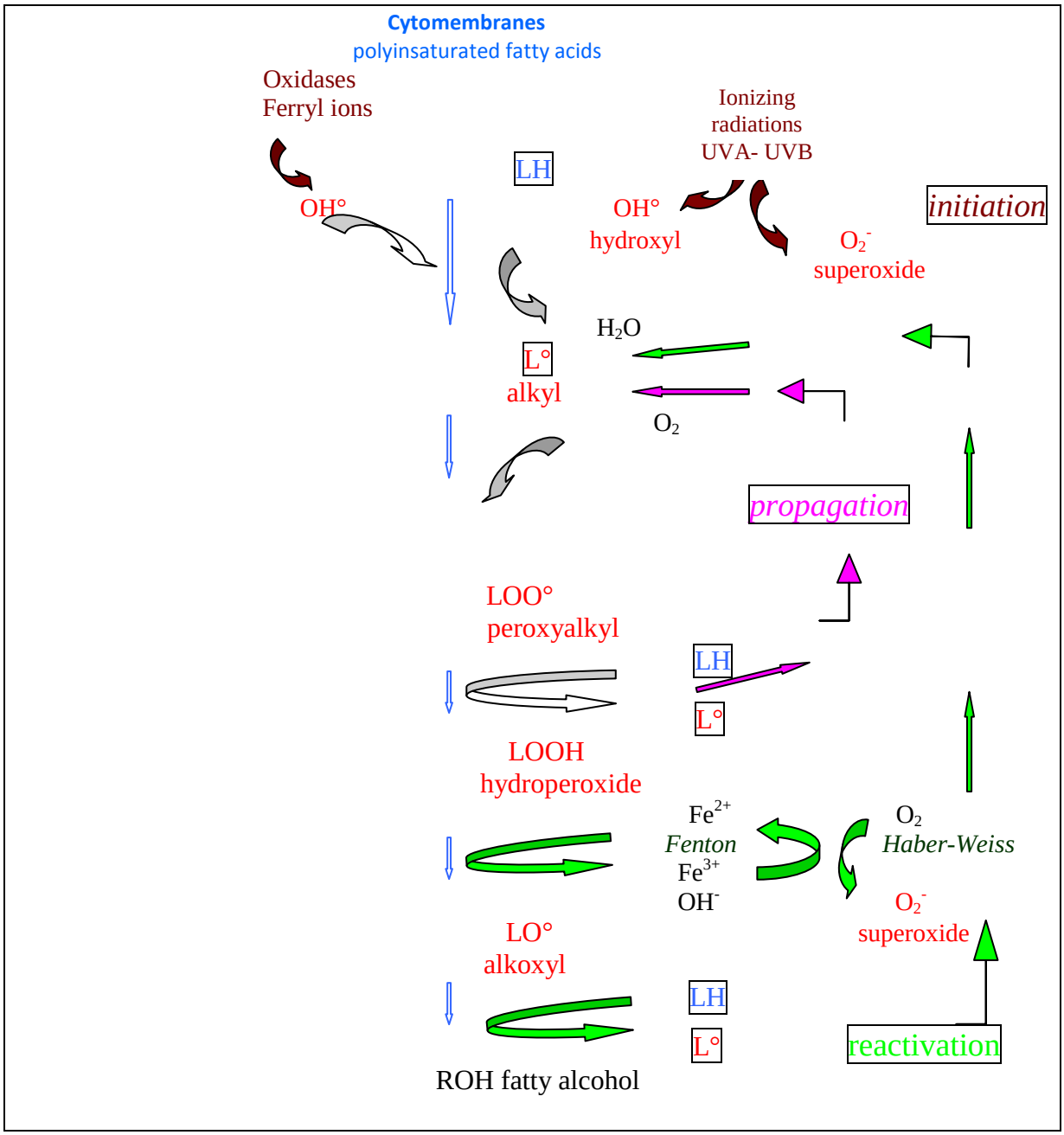

In order to observe if SEA HEATHER® is able to intercept one or several steps of the autoxidative system, cell cultivation is monitored.

Cultured human keratinocytes and fibroblasts (Line L929) are exposed to three different peroxidative systems which generate different reactive oxygen species:

- hypoxanthine-xanthine oxidase system which induces the generation of superoxide anion and hydrogen peroxide,
- aggression with t-butyl hydroperoxide which induces the generation of alkoxyl radicals,
- UVA irradiation in which several oxygen species are involved: *e.g.* singlet oxygen, hydroxyl radical and hydrogen peroxide.

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SEA HEATHER® is added in the respective cell culture medium according to several ways :

- ➲ P1 : before radical aggression during 24h (absent during aggression),
- ➲ P2 : during radical aggression (duration variable),
- ➲ P3 : before and during radical aggression.

Obtained effects are evaluated using the LDH assay which is able of quantifying membrane alterations. Experimental conditions are detailed in front of each treatment results.

The protective activity is determined by the equation:

$$C = [(A-B)/A] \times 100$$

where : $A = [(OD\ cw - OD\ ew) / OD\ cw] \times 100$

$B = [(OD\ tcw - OD\ tew) / OD\ tcw] \times 100$

OD : optical density, cw: control wells, ew: exposed wells , tcw: treated control wells, tew: treated exposed wells.

Protection against superoxide anion & hydrogen peroxide

In this peroxidative system, cytotoxicity is dependent upon hydrogen peroxide which may penetrate cells and react with endogenously activated oxygen and generate toxic compounds (Noel-Hudson *et al.*, 1989 – Toxic *In vitro* 3:103-109 ; 1990 – Int. J. Cosm. Science 12:105-114).

Superoxide may act as either an oxidant or a reductant. It can oxidize numerous molecules such as ascorbic acid. It can reduce others, specially cytochrome C and metals ions.

A dismutation reaction leading to the formation of hydrogen peroxide and oxygen can occur spontaneously or be catalysed by the enzyme superoxide dismutase.

Method

Culture methods used are adapted from the paper of Noël-Hudson *et al.,* (1990- Int. J. Cosm.SC., 12:105-114).

Cells are cultured in their respective media which is changed every two days. Reaching confluence, they are trypsinated and seeded into 96-well microplates maintained in a 5% CO^2 atmosphere at 37°C.

After 48h cultivation in microplates, media are removed. Cells are washed with PBS solution and then exposed to free radical generating system. Hypoxanthine and xanthine oxidase are diluted in PBS and used at the following concentrations – HX: 160 $\mu g.ml^{-1}$ for both cell types and XO: 20 $mU.ml^{-1}$ for keratinocytes and $5mU.ml^{-1}$ for fibroblasts. The duration of aggression is 150 min for both cell types.

The algal extract (2 and 4%) is added before aggression for 48 h (P1- aggression without agent) and before and during aggression (P3). Alpha-tocopherol is first diluted in 5% ethanol ($1x\ 10^{-2}M$) then added at $5.10^{-4}M$.

After 150 min, the aggression is stopped by washing cells with PBS. The LDH assay (Kit Sigma) is performed on the culture supernatant immediately after.

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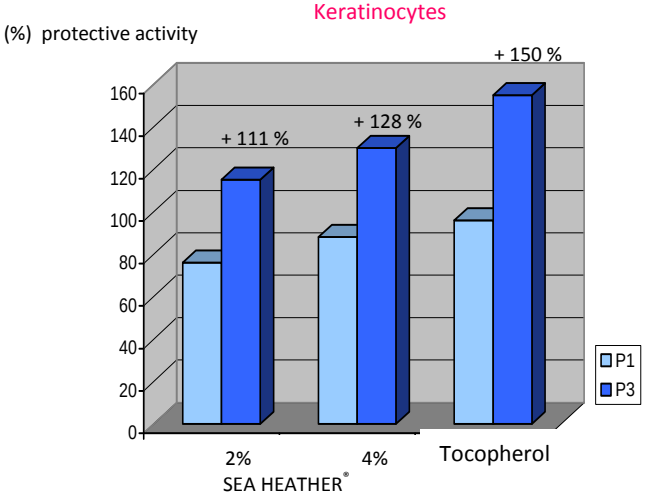
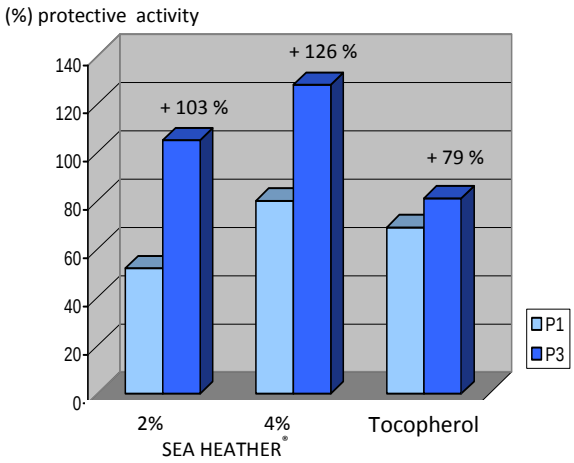
Results

All results represent the average of 12 values for each concentration ($\alpha = 0.05$).

Keratinocytes



Fibroblasts



➤ The protective effects of SEA HEATHER® against superoxide anion involve that the initiation step and the Haber-Weiss cycle of the autoxidative processes are stopped.

Protection against alkoxyl radical

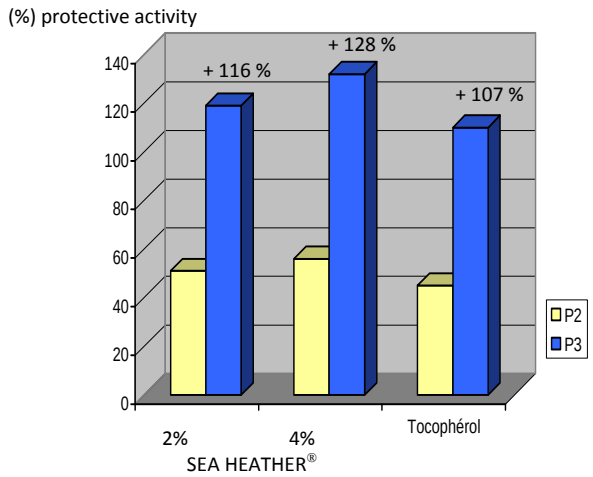
Alkokyl radical is considered as a radical with medium oxidant ability towards lipids. However, it allows the propagation of the lipid peroxidation reaction by dismutation of peroxides.

Method

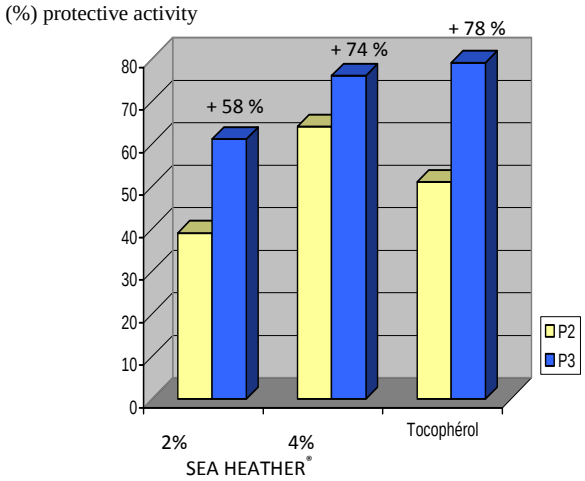
In the present treatment, the aggression is carried out with t-butyl hydroperoxide in presence of ferrous ions. The duration is 7 h for keratinocytes and 6 h for fibroblasts.
The algal extract (2 and 4%) and alpha-tocopherol (5.10^{-4}M) are added either during aggression (P2) or before (during 48 h.) plus during aggression (P3). The LDH assay is performed on culture supernatants immediately after washing cells with PBS solution to stop attack.

Results

Keratinocytes



Fibroblasts



➤ The protective effects of SEA HEATHER® against alkoxyl radical imply that the reactivation step of the autoxidative processes is stopped

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Protection against UVA

Intracellular release of hydroxyl radical & singlet oxygen

Hydroxyl radical is the most powerful oxygen radical in biological systems. In the autoxidative system of lipoperoxidation, its role is analogous to a "spark" that starts a fire. It is able to start a chain reaction with an extreme reactivity. It is generated from Fenton and Haber-Weiss reactions.

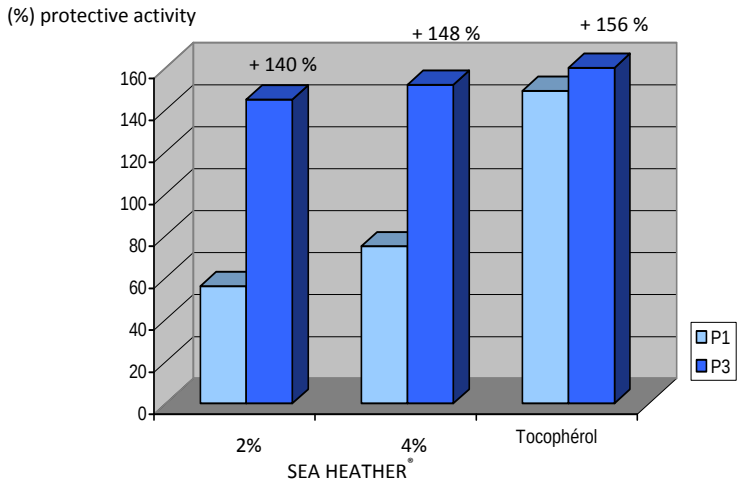
Singlet oxygen may react readily with unsaturated fatty acids producing a complex mixture of hydroperoxides.

Method

Fibroblasts (Line L929) seeded into 96-well microplates are irradiated with UVA (dose: 24 $J.cm^{-2}$) in PBS solution.
The algal extract (2 and 4%) and tocopherol (5.10^{-4}M) are added either before aggression in the culture medium (P1, aggression without SEA HEATHER®) or before aggression in the culture medium plus during aggression in PBS solution (P3).
The LDH assay is performed on culture supernatants immediately after washing cells with PBS solution to stop attack.

Results

All results represent the average of 12 values for each concentration ($\alpha = 0.05$).



➤ SEA HEATHER® protects cell membranes against lipoperoxidation caused by UVA irradiation.

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In summary concerning the autoxidative system of the lipoperoxidation

➤ SEA HEATHER® is able of

- penetrating cells (keratinocytes and fibroblasts) and inhibiting reactive oxygen species of any nature and origin,
- stopping the synthesis of the most highly reactive oxygen species : hydroxyl and alkoxyl radicals,
- blocking the autoxidative chain reactions on three levels (initiation step, Haber-Weiss cycle, reactivation step).

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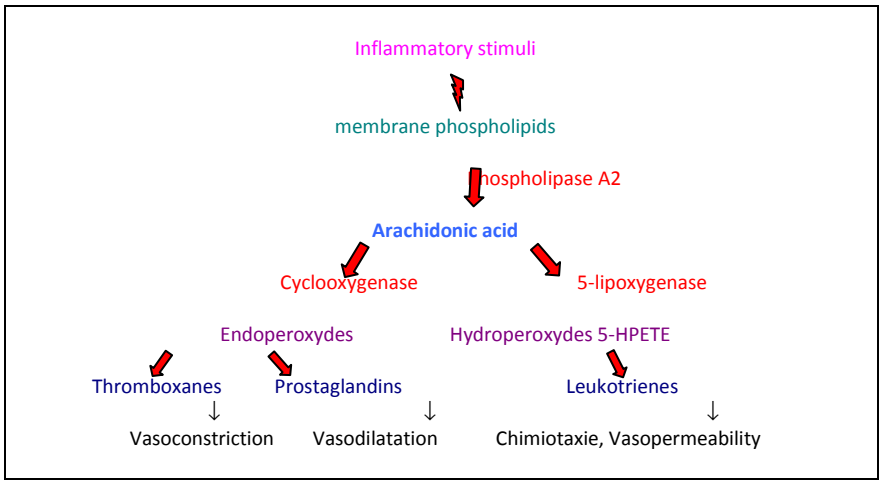
Protection against inflammation

In cell membranes, phospholipids may be also altered by the enzymatic pathway of the lipoperoxidation which induces the arachidonic acid cascade (*cf.* diagram below).



The arachidonic acid is released from membrane phospholipids upon cell stimulation through the action of phospholipases (A2 and C). Then, it is converted into bioactive substances according to two pathways :

1- through the activity of 5-lipoxygenase: the oxidized compounds formed are hydroperoxydes which are the precursors of leukotrienes,

2 - through the activity of cyclooxygenase: the oxidized compounds formed are endoperoxydes which are the precursors of thromboxanes and prostaglandins.

In the present experiment, only the first pathway is taken into consideration.

Lipoxygenases catalyze the epoxidation of unsaturated fatty acids and their esters to produce leukotrienes *via* the formation of hydroperoxydes. They are involved in a wide range of diseases (Tang *et al*., 1994- Cancer Res., 54:1119-1129; Sigal *et al*., 1994 – Ann. N.Y. Acad. Sc., 714 : 211-224).

Leukotrienes have potent biological actions, such as degranulation and plasma exudation (Samuelsson *et al*., 1987 – Science, 237: 1171-1176).

They also participate in host defence reactions and pathological conditions, such as immediate hypersensitivity and inflammation.

The following experiments show that SEA HEATHER® presents

- anti-inflammation properties as proved by spectrophotometric assays,

- anti-irritation properties as illustrated both by cytological observations performed on reconstituted human epidermis and a clinical study based on the stinging test.

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Inhibition of the phospholipase A2

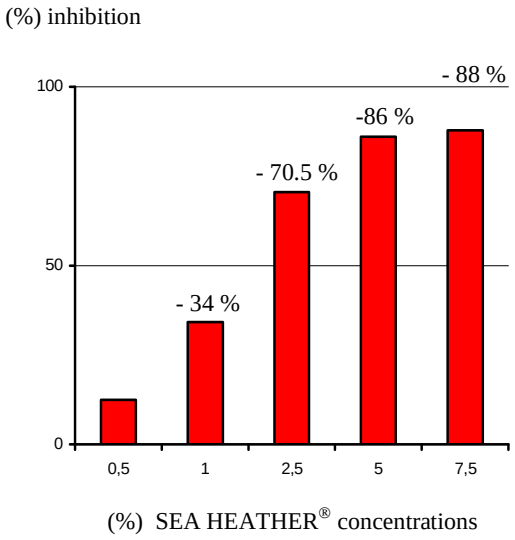
The enzyme phospholipase A2 plays a central role in the release of the arachidonic acid from membrane phospholipids upon cell stimulation.

Methods

This method uses a phospholipid (dimyristoyl L-phosphatidylcholine) as the substrate. In presence of the enzyme, lysolecithine is formed with liberation of an insoluble fatty acid into the reactionnel medium.The reaction is followed by turbimetry using a spectrophotometer at 360 nm.

Results

Results are expressed in % of anti-phospholipase activity comparatively to control realized without SEA HEATHER®. They represent the average of three experiments for each concentration tested ($\alpha = 0.05$).



Inhibition of the 5-lipoxygenase

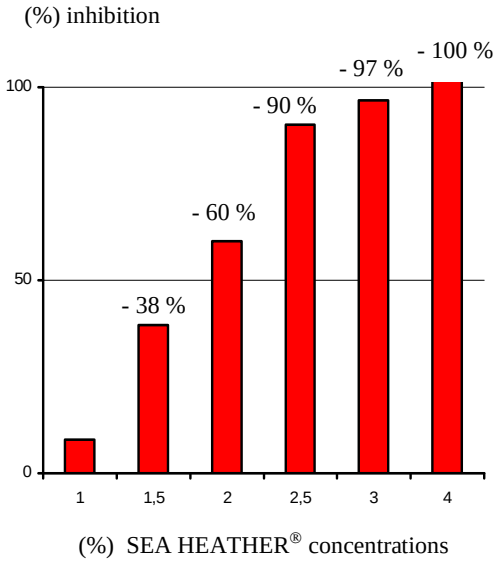
Lipoxygenases catalyze the oxidation of the arachidonic acid to bioactive lipid hydroperoxides.

Methods

This method uses an insaturated fatty acid (the linoleic acid) as the substrate. In presence of the enzyme, there is formation of a peroxide (5 HPETE: 5-hydroperoxy-6,8,11,14-eico-satetraenoïc acid).
The enzyme reaction is monitored at 234 nm until the reaction rate reached a steady state. This wavelength corresponds to the absorption of the hydroperoxides generated by the action of the lipoxygenase on linoleic acid.

Results

Results are expressed in % of anti-lipoxygenase activity comparatively to control realized without SEA HEATHER®. They represent the average of three experiments for each concentration tested ($\alpha = 0.05$).



➤ SEA HEATHER® acts within the arachidonic acid cascade at two levels with dose- dependent effects. It inhibits the activity of phospholipase A2 and stops the release of the arachidonic acid. It also inhibits the activity of 5- ipoxygenase that will inhibit the production of leukotrienes.

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Anti-irritation properties

Protection of cell membrane functionality after irritation

Transmission electron microscope observation of reconstituted skins submitted to irritant stress

The fine structure of epidermal cells is observed to examine eventual changes in response to dimethyl sulphoxide exposition. DMSO produces irritation characterized by a burning sensation.

Methods

Several experiments are performed on reconstituted human epidermis (model SkinEthic-17 days) :

- experiment n°1 : cultivation during 24 h in absence of SEA HEATHER®, treatment with DMSO (10% in the growth medium for 6 h.),

- experiment n°2 : cultivation during 24 h in presence of SEA HEATHER® at the concentration of 4% in the growth medium, treatment with DMSO (10% in the growth medium for 6 h.),

- experiment n°3 : cultivation in absence of both SEA HEATHER® and DMSO treatment.

After 24 h cultivation at 37°C, samples are prepared for electron microscope observations.

They are fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4 for 2 h30 at 4°C. After washing in buffer, they are post-fixed in 2% osmium tetroxide for 2 h. in the same buffer and temperature.

Following dehydration through a graded ethanol series with a final treatment in absolute ethanol, samples are embedded in a Spurr resin.

Sections cut on a Reichert UltraCut E ultramicrotome are post-stained in uranyl acetate and lead citrate.

Micrographs are taken using Zeiss EM 912 transmission electron microscope.

Results

Results are grouped on two plates: one (p.17) concerns the controls in presence and absence of DMSO and absence of SEA HEATHER®, the other (p.18) concerns the epidermis submitted to DMSO treatment in presence of SEA HEATHER®.

Micrographs represent superficial cell layers.

- DMSO treatment induces important cytological perturbations with cytoplasm alterations and large dilations in intercellular spaces.

- These perturbations are not visible on the micrographs relative to the experiment realized in presence of SEA HEATHER®.

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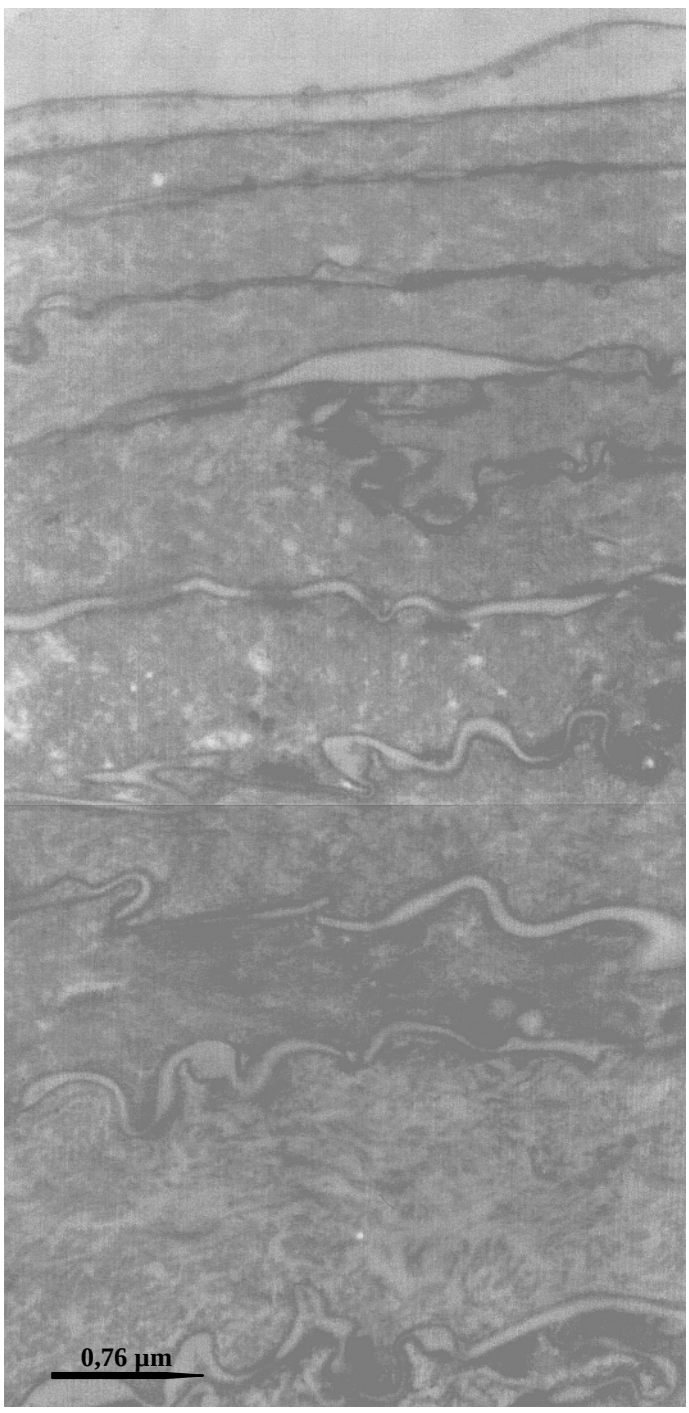
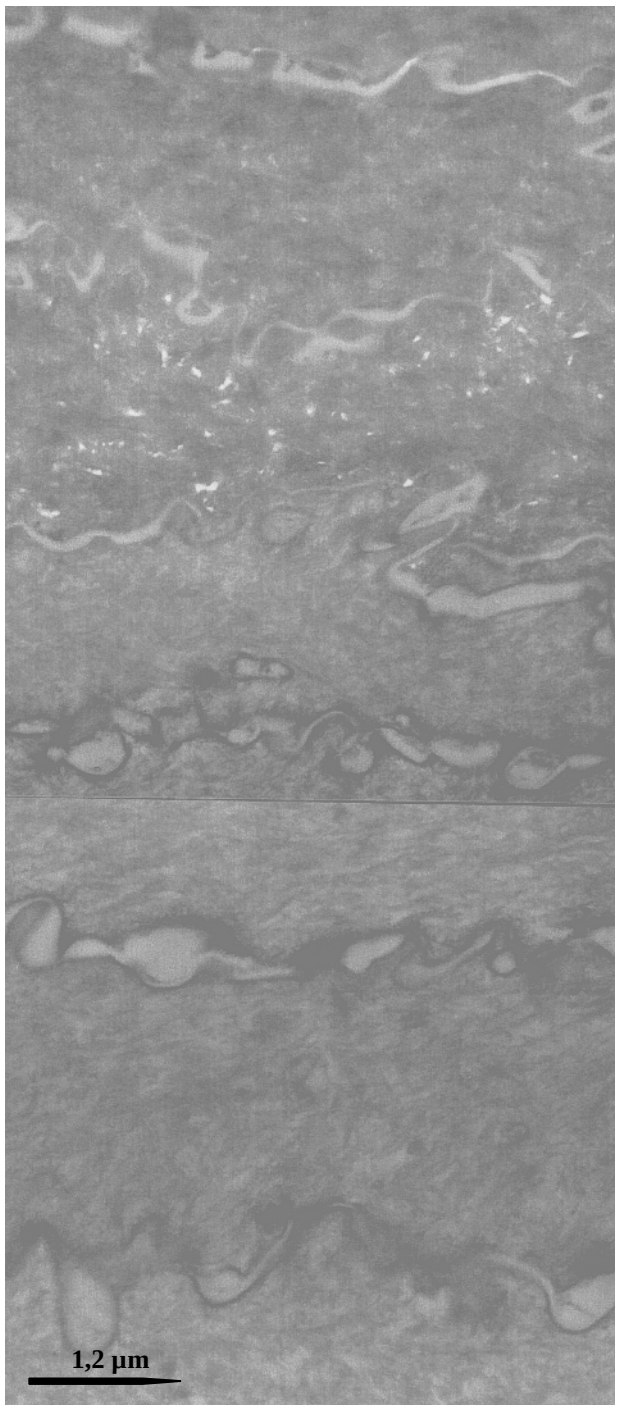
Control Without DMSO treatment	Control DMSO treatment for 6 hours





➤ DMSO treatment induces important ultrastructural alterations.

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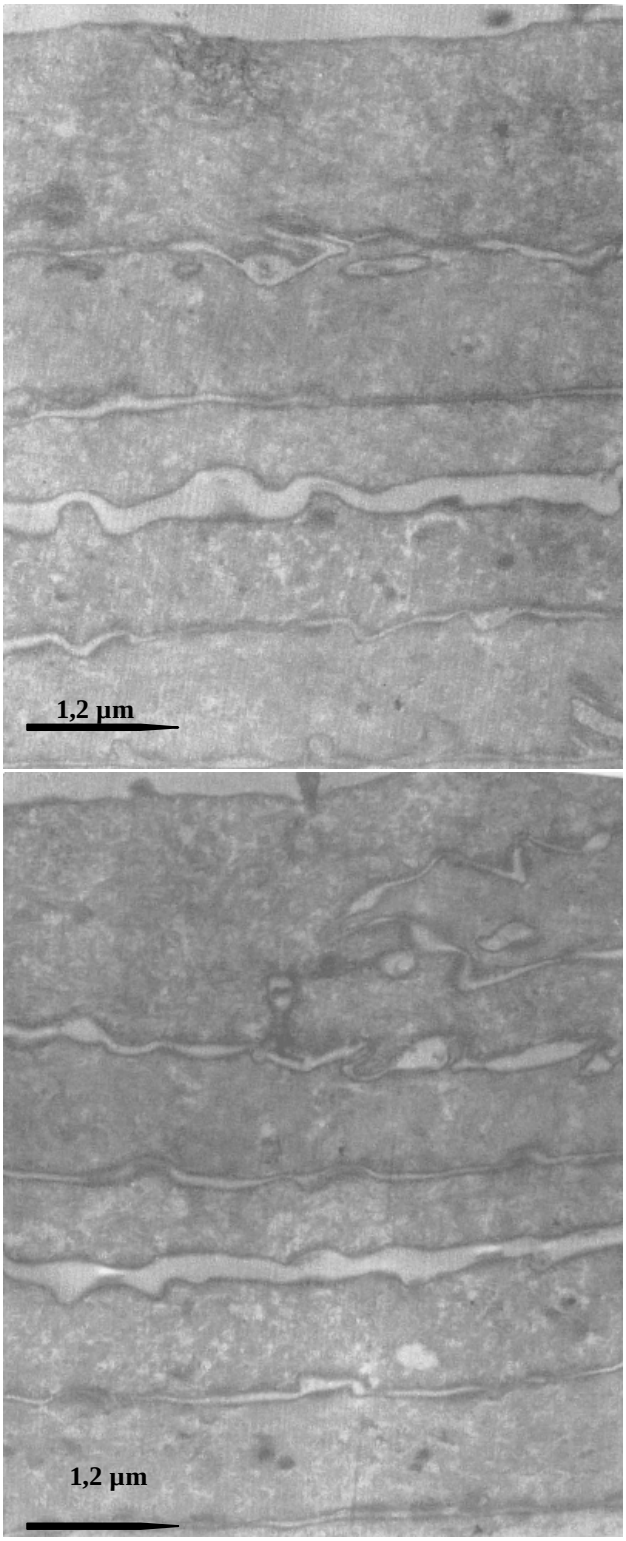
SEA HEATHER®
DMSO treatment for 6 hours



➤ The addition of SEA HEATHER leads to higher preservation of the fine cell structure, thus guarantees full cell membrane functionality after chemical-induced irritation.

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Reduction of stinging sensations

Clinical study

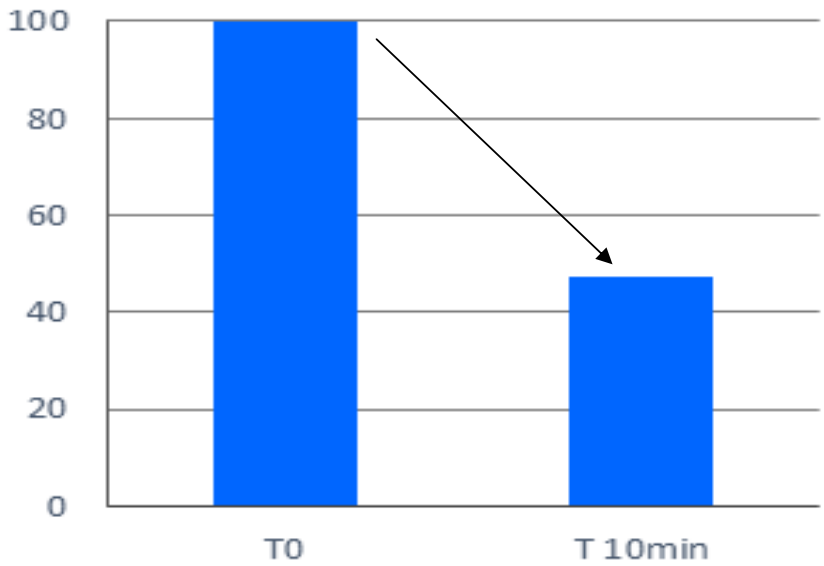
Methods

Evaluation of the anti-irritant properties of SEA HEATHER® incorporated at 10% into a Carbopol gel on the nasolabial fold of 22 volunteers (18 female - 4 male of 20-66 years old) after irritation induced by a solution 10% lactic acid (stinging test) (IDEA-FRANCE).

The activity is evaluated after treatment at 10sec – 2min30sec and 5 min.

Results

The topical use of a Carbopol gel added with 10% Sea Heather gives an average improvement of 53% (52.94%) against the stinging associated with the lactic acid test.



➤ SEA HEATHER® gives efficient soothing activity on irritated skin.

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CONCLUSION & COSMETIC BENEFITS

SEA HEATHER® is a patented active ingredient derived from the Mediterranean brown alga described as caespitose *Cystoseira species*.

SEA HEATHER® works through mechanisms which prevent the lipid peroxidation and inflammatory responses.

Its ability to protect DNA against damage caused by singlet oxygen has been demonstrated using the 3D assay. 50% protection are reached with a dose of 0.2% only.

SEA HEATHER® can enter cells and inhibit various reactive oxidative species involved in the non enzymatic pathway of the lipid peroxidation, especially the most reactive species: hydroxyl and alkoxyl radicals.
On that account, it protects against the lysis of cutaneous cells (keratinocytes and fibroblasts) against the release of radicals during this pathway. It is able of penetrating cells and inhibiting reactive oxygen species of any nature and origin. It is also able of blocking the chain reaction of oxidation on three levels (initiation step, Haber-Weiss cycle, reactivation step).

SEA HEATHER® also prevents inflammation by inhibiting of both

- phospholipase A2, (thereby limits the production of arachidonic acid) and
- 5-lipoxygenase, (thereby limits the production of leukotrienes).

SEA HEATHER® offers very good protective, anti-inflammatory properties and anti-irritation properties too as proved by electron microscopic studies performed on reconstituted human epidermis and confirmed by sting test on volunteers.

This makes SEA HEATHER® an excellent ingredient for products aimed at helping to reduce irritation, soothing the skin and improving the comfort of reactive and sensitive skins.

COSMETIC APPLICATIONS

SEA HEATHER® can be incorporated into all products for:

- Soothing care for reactive and sensitive skins.
- Protecting care for irritated, sunburnt, razor burnt skins.
- Daily protecting care.
- Anti-aging care.
- Sun and after sun care.
- Scalp care.

Recommended use levels: 2 - 10**%.**

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ANNEX

Evaluation of ocular irritation

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N° Etude: 191891F01.doc
Version :N° 1
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STUDY SUMMARY

EVALUATION OF THE POTENTIAL IRRITANCY OF A PRODUCT THROUGH ITS APPLICATION ON THE CHORIOALLANTOIC MEMBRANE OF A CHICKEN EGGSHELL: *Het Cam Method*

- **Tested product** : SEA HEATHER
- **Promoter** : GELYMA
- **Objective**: To assess the irritant potential of the tested product
- **Methodology**: The principle of this study is based on the visual observation, by a trained person, of the possible irritations (hyperaemia, haemorrhaging, coagulation / thrombosis) that may appear during the five minutes that follow the application of the said product on the chorioallantoic membrane of an embryonic chicken egg after eleven days of incubation.
- **Dates of study :** 12/12/2006
- **Place of study:** EUROFINS ATS, Pôle d'activité d'Aix en Provence
  Actimart, 1140, rue Ampère,
  13851 AIX EN PROVENCE cedex 3
- **Results :**

Denomination	ATS Reference	Initial concentration	Results	
			Score	Classification
SEA HEATHER	167111	100%	2.5	Slightly irritant

- **Conclusion :**

According to the performed experimental conditions, the product SEA HEATHER tested by the HET CAM method, at 100 %, can be considered as slightly irritant regarding its ocular primary tolerance.

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TEL +33 (0)4.42.39.78.08 - FAX +33 (0)4.42.39.77.81
N° SIRET : 33761796300067 – Code APE : 743 B

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Evaluation of cutaneous irritation

eurofins | ATS

N° Etude: 191891F02.doc
Version : N° 1
Page : 15
P05.0.DOC.00017.01

STUDY SUMMARY

EVALUATION OF THE CUTANEOUS TOLERANCE OF A COSMETIC PRODUCT AFTER A SINGLE APPLICATION UNDER OCCLUSIVE PATCH DURING 48 HOURS:
Patch test method

- **Product tested** : SEA HEATHER
- **Promoter** : GELYMA
- **Monitor** : Liliane PELLEGRINI, R & D Manager
- **Objective** : Assessment of the cutaneous local tolerance of the studied product after an epicutaneous test performed in occlusive conditions, during 48 hours.
- **Place of the study:** EUROFINS SCIENTIFIC TEST CENTER,
  3 allée des Ingénieurs
  1140 rue André Ampère
  13851 AIX EN PROVENCE cedex 3
- **Investigator** : Doctor Mary CREST
- **Date of study:** from 28/11/06 to 30/11/06 and from 12/12/06 to 14/12/06
- **Methodology:**
  - ✓ ***Application modes:***

Area of application : on the back
Quantity of product : 0.02 ml
Frequency and duration : only one application during 48 hours
Conditions of application : product applied pure under occlusive patch.

- ✓ ***Assessment method:***

A dermatologist makes the clinical observation, after the removal of the patches. The quantification of the cutaneous irritation is given according to a numeric scale (rash, oedema, dryness, blister). The average irritant score of the product to be tested is measured with the average of the quotations obtained for the whole volunteers, allowing ranking the product from "not irritant to very irritant". The assessment is always made by comparison with the "negative" control: patch alone.

- **Population:** 11 healthy adult volunteers.
- **Results:** The average irritant score of the product is 0,0.
- **Conclusion:**

According to the experimental conditions taken into account, after only one application of 0.02 ml of product, under occlusive patch and during 48 hours, on 11 healthy adult volunteers, and according to the scale used for the interpretation of the results, the raw material **"SEA HEATHER", Lot 06 04 140, can be considered as not irritant regarding its primary cutaneous tolerance.**

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Evaluation of genotoxic effects

Method

This chemiluminescent 3D Assay is an ELISA-like assay, realized by the well known company S.F.R.I.(St Jean d'Illac, France), by using plasmid DNA adsorbed on sensitized microplates as the substrate.

This method is based on a repair reaction of DNA (Salles *& al*., 1995 – Analytical Biochemistry 232:37-42; Patent FR n° 95003230).

DNA lesions are repaired by the excision repair pathway which implies an incision-excision reaction followed by DNA repair synthesis.

In the present experiment, these lesions were performed by singlet oxygen generated by methylene blue (10 µg/ml in extrapure water).

SEA HEATHER® is added according to 4 concentrations: 10 – 1 – 0.1 and 0.01%. The positive standard is MMS.

Results

The ability of a molecule to alter DNA is measured by the reparation ratio R .

R = RLU sample at a known dilution / RLU solvent alone
RLU: Relative Light Units

When R is inferior to 2, there is no genotoxicity,

When R is superior to 2, there is a significant genotoxicity.

Results represent the mean of two independent experimentations. They are expressed comparatively to control (irradiated or no-irradiated solvent).

Product	Concentrations	Ratio of genotoxicity
SEA HEATHER®	10	0.29
	1	0.51
	0.1	0.87
	0.01	0.84
Positive standard (MMS)	5mM	2.71
	2mM	2.02

➤ No direct genotoxicity *in vitro* with the used conditions.

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