(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 107 723 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
26.11.2003 Bulletin 2003/48

(21) Application number: **99941803.1**

(22) Date of filing: **05.09.1999**

(51) Int Cl.[7]: **A61K 7/00**, A23L 1/303, A61K 7/06, A61K 7/42, A61K 7/48

(86) International application number:
PCT/IL99/00478

(87) International publication number:
WO 00/013654 (16.03.2000 Gazette 2000/11)

(54) **A CAROTENOID COMPOSITION CONTAINING PHYTOENE AND PHYTOFLUENE**

EINE PHYTOEN UND PHYTOFLUEN ENTHALTENDE CAROTENOIDE ZUBEREITUNG

PREPARATION A BASE DE CAROTENOIDES

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **04.09.1998 IL 12607698**

(43) Date of publication of application:
20.06.2001 Bulletin 2001/25

(73) Proprietor: **I.B.R. Israeli Biotechnology Researc, Ltd.**
62301 Tel Aviv (IL)

(72) Inventors:
- **SOUDANT, Etienne**
  F-94260 Fresnes (FR)
- **BEZALEL, Lea**
  84359 Beer Sheva (IL)
- **SCHICKLER, Hedva**
  76804 Mazkeret Batya (IL)
- **PALTIEL, Judith**
  76000 Rehovot (IL)
- **BEN-AMOTZ, Ami**
  56542 Savion (IL)
- **SHAISH, Aviv**
  79800 Shikmim (IL)
- **PERRY, Inon**
  62301 Tel Aviv (IL)

(74) Representative: **Desaix, Anne**
Ernest Gutmann - Yves Plasseraud S.A.
3, rue Chauveau-Lagarde
75008 Paris (FR)

(56) References cited:

EP-A- 0 769 551
WO-A-96/39869
FR-A- 2 620 131
FR-A- 2 759 904
US-A- 5 310 554
WO-A-93/04598
DD-A- 246 565
FR-A- 2 678 946
US-A- 4 199 895
US-A- 5 545 816

- **TONUCCI, L.H. ET AL.: "Carotenoid Content of Thermally Processed Tomato-Based Food Products" J. AGRIC. FOOD CHEM., vol. 43, no. 3, 1995, pages 579-586, XP002124250**
- **CHOO, Y.M.: "Production of palm oil carotenoid concentrate and its potential application in nutrition" STN INTERNATIONAL; FILE CHEMICAL ABSTRACTS, AN119:124982,1992, XP002124251 & LIPID-SOLUBLE ANTIOXID. EDITOR(S): ONG, AUGUSTINE S.H.; PACKER, LESTER. PUBLISHER: BIRKHÄUSER, BASEL, SWITZ,1992, pages 243-254,**
- **PATENT ABSTRACTS OF JAPAN vol. 005, no. 168 (C-077), 27 October 1981 (1981-10-27) & JP 56 099405 A (NISSHIN OIL MILLS LTD:THE), 10 August 1981 (1981-08-10)**
- **BEN-AMOTZ, A. ET AL.: "The biotechnology of cultivating Dunaliella for production of beta-carotene rich algae" BIORESOURCE TECHNOLOGY, vol. 38, 1991, pages 233-236, XP000901780**
- **BEN-AMOTZ, A. ET AL.: "Massive accumulation of phytoene induced by norflurazon in Dunaliella bardawil (chlorophyceae) prevents recovery from photoinhibition" JOURNAL OF PHYCOLOGY, vol. 23, no. 1, March 1987 (1987-03), pages 176-181, XP000901498**

Printed by Jouve, 75001 PARIS (FR)

(Cont. next page)

- **VAISBERG, A. J. AND SCHIFF, J. A.: "Events surrounding the early development of Euglena chloroplast" PLANT PHYSIOLOGY, vol. 57, 1976, pages 260-269, XP000901340**

Description

FIELD OF THE INVENTION

[0001] The present invention relates to a carotenoid comprising preparation. The preparation of the invention can be used in food, in cosmetics, in a variety of medicinal uses, etc. The invention also concerns a method for the production of the carotenoids.

BACKGROUND OF THE INVENTION

[0002] Carotenoids are pigments produced by microorganisms, fungi and plants, and used by them as antioxidants and protectants against excessive radiation. The most widely used carotenoids in food, medical preparations or cosmetics are β-carotene and lycopene. β-Carotene and lycopene are sensitive to light and oxidation, a property which considerably limits their use and shortens shelf-life of products containing them (in: Carotenoids, Chemistry and Biology, Krinski, N.I., Matthews-Roth, M.M., Taylor, R.F., (Eds), Planum Press, New York, London, 1989). In addition, β-carotene and lycopene have a distinctive orange color and this color has a serious limitation for a variety of cosmetic or food applications.

[0003] Phytoene (7, 8, 11, 12, 7', 8', 11', 12'-octahydro-γ,γ-carotene) and phytofluene (15Z, 7, 8, 11, 12, 7', 8'-hexahydro-γ,γ-carotene), are carotenoids (C-40 isoprenoids chain) which are precursors in the biosynthetic pathway which leads to the production of β-carotene, lycopene and other carotenoids (phytoene is the first carotenoid-specific precursor and phytofluene is produced therefrom in a subsequent desaturation step). Phytoene is completely colorless whereas phytofluene has a slight yellowish color. Japanese Patent Application No. 90-40520, disclosed that the introduction of a DNA sequence yielding expression of phytoene into certain transfected cancer cells, resulted in inhibition of their growth and in inhibition in the activation of Epstein Barr virus (EBV).

[0004] Compositions comprising phytoene and phytofluene are known from the prior art, see eg. LH Tonucci et al, J. Agric. Chem. 1995, 43, 579-586.

SUMMARY OF THE INVENTION

[0005] In accordance with the present invention it was shown that phytoene and phytofluene possess anti-oxidative properties and furthermore are capable of absorbing ultra violet (UV) light. In addition, although having these properties, phytoene and phytofluene were found to be much more stable to oxidation than, for example, β-carotene. These findings led to the realization that phytoene and phytofluene in combination could be useful in the prevention of environmentally induced damage of various kinds.

[0006] Thus, in accordance with the invention there is provided a composition comprising, an amount of phytoene and an amount of phytofluene, effective in combination in the prevention of damage resulting from oxidation and from exposure to UV light.

[0007] In accordance with the invention, the term "*damage*" is to be understood as any damage resulting from a variety of oxidative agents such as oxygen itself, hydroxyl radical, hydrogen peroxide, other free radicals, ozone etc., or from any kind of harmful UV irradiation, such as UVA and UVB irradiation. The damage would depend upon the target for which the preparation is used. Thus, if the preparation is used on skin, damage may be any skin damage such as burns, blisters, damage appearing after chronic exposure to sun, e.g. premature aging of the skin, etc. When the preparation is used as a food preservative, such damage may be in the form of a decrease in product stability, chemical modification resulting for example in rancidity, accelerated aging, etc.

[0008] In accordance with a preferred embodiment of the invention there is provided a topical skin composition for protecting the skin against environmental hazards, comprising phytoene and phytofluene in an effective amount such that, in combination, these carotenoids exert an oxidation- protecting and UV-protecting effect on the skin.

[0009] The term "*environmental hazards*" relates to any environmental agent which can exert damage such as UV radiation or oxidative agents.

[0010] The term "*effective amount*" should be understood to mean an amount of phytoene and an amount of phytofluene which, when administered in combination, achieves the desired protective effect.

[0011] The phytoene and phytofluene in the composition of the invention may each be either in their *trans* or in their *cis* forms.

[0012] The weight ratio between the phytoene and phytofluene in the composition of the invention can range between 200:1 to 1:200, respectively, typically between about 50:1 to 1:50, preferably from 10:1 to 1:10, 10:1 (phytoene:phytofluene) being a particular example. The above ratios of phytoene to phytofluene may be reached either by using an extract which contains both carotenoids in the desired ratio, by adding an additional amount of one of the carotenoids to an extract comprising both carotenoids so that the desired ratio is obtained or by mixing the two separate carotenoids

(each obtained by any of the methods mentioned or described above and below) to reach the desired ratio between them.

[0013] One of the novel features of the inventive composition is that while possessing the above noted properties, the combination of phytoene and phytofluene is essentially devoid of any color (but for a slight yellowish hew, hardly visible, of the phytofluene). The fact that the composition is essentially colorless ensures that these carotenoids will not have any effect on the aesthetic properties of the preparation comprising them. In addition, the lack of absorbance of light in the visible range (which is a manifestation of the fact that they are essentially colorless) renders them stable to degradation under visible light.

[0014] The preparation may, in accordance with the above noted preferred embodiment, be used as a topical cosmetic or pharmaceutical preparation in order to protect the skin from environmental hazards such as those described above including UV (UVA and/or UVB) irradiation or damages which can be effected by a variety of oxidative agents. A topical composition of the invention may be in the form of a gel, an oil-in-water or water-in-oil emulsion, a salve or ointment, etc.

[0015] In accordance with another embodiment, the composition may be used as an additive in food preparations, e.g. serving as a preservative to protect against oxidation of the various food ingredients, e.g. of oils or fats.

[0016] At times, the composition of the invention may comprise additional components which do not substantively change the basic characteristics of the composition. One example of such a component is zetacarotene (7,8,7',8' - tetrahydro-γ, γ-carotene).

[0017] The composition of the invention may obviously, depending on its use, comprise also other ingredients, cosmetical or pharmaceutical acceptable carriers, preservatives, other antioxidants, various pharmaceutically or cosmetically active ingredients such as a topically acting drugs, etc.

[0018] In accordance with one preferred embodiment, the composition comprises also a hydrophobic carrier, which may be selected from oils typically used in the cosmetic, pharmaceutical or food industry, such as vegetable, mineral or synthetic oils.

[0019] The phytoene and phytofluene may be obtained from a variety of sources. Typically, they may be obtained from organisms that produce carotenoids, such as a variety of plants, various algae, and particularly micro algae *Dunaliella sp*, being a specific example. Very low amounts of phytoene and phytofluene have been produced by β-carotenoid producing organisms. To obtain even such low amounts of the carotenoids, the organisms were grown under dim light conditions. In accordance with the invention, a method is provided which enables to yield substantive amounts of at least one of phytoene or phytofluene from such carotenoid producing organisms. The term *"substantive amounts"* relates to an amount of phytoene or an amount of phytofluene ranging from about 0.1 mg/l culture to about 30 mg/l culture typically between about 1 mg/l to about 20 mg/l. When the carotenoid producing organism is the algae *Dunaliella sp.*, the typical amount of phytoene and phytofluene which may be produced is between about 1 mg/l to about 15 mg/l. In accordance with the method of the invention, the carotenoid producing organism, typically algae, may be grown under various kinds of conditions.

[0020] In accordance with one embodiment of this aspect of the invention, the carotenoid producing organisms are grown outside, for example, in the case of *Dunaliella sp.* in outside pools in a growth medium comprising sea water and recycled salt water at a temperature of between about 10°C to about 40°C. The organisms may be grown under sunlight at an extent of between 80% shade to full sunlight. By a preferred embodiment, the carotenoid producing organisms are grown under bright light ranging from about 40% shade to full sunlight.

[0021] By an additional embodiment, the carotenoid producing organisms are grown in a growth culture, typically, in a fermentor and typically at room temperature in which case the growth medium will typically comprise various salts and minerals in different combinations (see, for example, Example 1.1 below). In accordance with this embodiment, the organisms are grown under broad spectrum light (light spanning over a substantive portion of visible light) of above about 10 W/m^2 and which may range from about 10 W/m^2 to about 200 W/m^2.

[0022] In accordance with the method of the invention, substantive amounts of phytoene and/or phytofluene are typically produced following several days of growth of the organism (e.g. about 4 to 6 days).

[0023] The phytoene and phytofluene are obtained under conditions which favor the accumulation of phytoene and phytofluene in cells of the organisms, typically by growing a β-carotene-producing organism in the presence of carotenoid-biosynthesis inhibitors, which are inhibitors that can act in subsequent reaction steps in the biochemical pathway of the production of carotenoids. One example of inafter: *"the 4-chloro inhibitor*"). When the source of phytoene and phytofluene is the micro algae *Dunaliella*, the 4-chloro inhibitor is typically included in the growth medium in a concentration of about 0.1 μM (Ben-Amotz *et al., Plant Physiol*. **86**:1286, 1988). Other inhibitors which may be used are, for example, J334, (Ben-Amotz *et al. Supra*) Sandoz H 6706 (Kümmel, H.W. *et al*., Z. Naturforsch 30c, 333, 1975), diphenyl-amine (DPA) (Foppen F.H., *Ann. Ist. Super. Sanita*, 439, 1969) and nicotine (Shaish *et al, Plant Cell Physiolo* **31**:689, (1990)).

[0024] In accordance with one embodiment of this aspect of the invention there is thus provided a method for preparing a composition comprising at least one of phytoene or phytofluene, comprising the steps of:

(i) incubating a carotenoid producing organism in a growth culture under broad spectrum light having an intensity of above about 10 W/m^2 in the presence of one or more carotenoid synthesis inhibitors;
(ii) growing said carotenoid producing organisms until the level of at least one of phytoene or phytofluene is between about 1 mg/l culture to about 50 mg/l culture; and
(iii) separating said organisms from the culture.

[0025] In accordance with an additional embodiment of this aspect of the invention there is provided a method for preparing a composition comprising at least one of phytoene or phytofluene, comprising the steps of:

(i) Incubating a carotenoid producing organism in a growth culture under sunlight at an extent between about 80% shading to full sunlight in the presence of one or more carotenoid synthesis inhibitor;
(ii) Growing said carotenoid producing organisms until the level of at least one of phytoene or phytofluene is between about 1 mg/l culture to about 50 mg/l culture; and
(iii) separating said organisms from the culture.

[0026] In accordance with this aspect of the invention, the phytoene and phytofluene obtained in the grown organisms may be used without being separated

[0027] In accordance with this aspect of the invention, the phytoene and phytofluene obtained in the grown organisms may be used without being separated from the dry matter of the producing organism. However, in accordance with a preferred embodiment, following separation of the carotenoid producing organism from the culture, a preparation is further prepared from the organisms comprising phytoene, phytofluene or both. Typically, the preparation is an extract which may be prepared from the carotenoid producing organisms by any of the extraction methods known in the art such as, for example, by ethanol:hexane extraction.

[0028] In accordance with the method of the invention, typically, both phytoene and phytofluene are obtained from the carotenoid producing organism and the ratio of phytoene to phytofluene may vary according to the conditions under which the organism was grown and, when a preparation is prepared, the type of methods used for obtaining the preparation. At times, however, under certain conditions, growing the carotenoid producing organism in accordance with the method of the invention may result in obtaining mostly one or only one of the two carotenoids.

[0029] In accordance with the invention it was also found that the carotenoids ratio may further be enhanced in favor of phytoene and phytofluene by the addition of active charcoal during carotenoid extraction. Charcoal may be added when an inhibitor is used as well as when the carotenoid producing organism is grown without the inhibitor. The charcoal is later filtered out from the culture or preparation using any of the known centrifugation or filtration methods.

[0030] In addition to the above, in accordance with the invention the carotenoids in the composition of the invention may also be synthesized by any of the known chemical or biochemical methods or recombinant methods. Chemically, phytoene can be synthesized, for example, from two geranylgeranyl pyrophosphates (C-20), in a reaction which may be mediated by phytoene synthase. The geranylgerranyl pyrophosphate can be obtained directly, by the conversion of mevalonic acid or by the condensation of pyrovate and glyceraldehyde-3-phosphate. Phytofluene can be synthesized by desaturation of phytoene, a reaction which may be mediated by phytoene desaturase. Recombinant methods include, for example, the mutagenesis of enzymes which are active downstream to phytofluene. Such synthesized phytoenes and phytofluenes will have activities which are substantively similar to the activities of the phytoene and phytofluene obtained from organisms that produce carotenoids as explained above.

[0031] The stability of the carotenoids in the composition of the invention can be tested by irradiating the carotenoid composition by light and/or exposing the composition to oxygenating agents. The effect of such treatments on the carotenoids in the compositions of the invention, is less than the effect of such treatments on other carotenoids such as, for example, β-carotene. In prior art compositions containing an effective amount of carotenoids, such treatment typically result in degradation of the carotenoids, manifested for example by change in light absorbance, i.e. color. The anti-oxidative effect of the composition of the invention can be determined, for example, by determining the anti-oxidation protection effect on DNA (Salles *et al., Anal. Biochem.*, **232**:37, 1995).

[0032] The invention will now be illustrated further in the following description of the specific embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS:

[0033]

Fig. 1 shows the UV absorbance spectra of a composition comprising phytoene extracted from *Dunaliella sp.* grown with the 4-chloro inhibitor.
Fig. 2 shows the UV absorbance spectra of phytofluene extracted from *Dunaliella sp.* grown with the 4-chloro inhibitor.

EXAMPLES

I. MATERIALS AND METHODS

1. Growth mediums

[0034] The following growth mediums were used:

1.1 A growth medium comprising:

$MgSO_4$, 5 mM; $CaCl_2$, 0.2 mM; KH_2PO_4, 0.2 mM; $FeCl_3$ + Na_2EDTA, 2 μM+5 μM; $MnCl_2$, 7 μM; $CuCl_2$, 1 μM; $ZnCl_2$, 1 μM; $CoCl_2$, 1 μM; $(NH_4)_6MO_7O_{24}$, 1 μM; NaCl, 1M; $NaHCO_3$, 50 mM; KCl, 5 mM.

The growth medium is typically used for growth of carotenoid producing organisms inside under artificial conditions.

1.2 A growth medium comprising sea water and recycled salt water in an amount which results in 2M salinity. Such a medium is typically used while growing a carotenoid producing organism outside under sunlight. The final pH of the growth mediums was 7.0 to 8.0.

2. Chemicals

[0035] β-Carotene biosynthesis inhibitor 4-chloro-5(methylamino)-2-(3-(trifluoromethyl)phenyl)-3(2*H*)-pyridazinone. The 4-chloro inhibitor was used for phytoene and phytofluene production. The range of concentration of the 4-chloro inhibitor was between about 0.07 μM to about 0.5 μM.

[0036] Untreated active charcoal was used to increase the ratio of phytoene and phytofluene to other carotenoids. The charcoal was filtered out from the extract at the final steps of preparation of the extract.

[0037] Various commercially used oils were used to dissolve the phytoene and phytofluene.

3. Growth of *Dunaliella sp.*

[0038] The algae *Dunaliella sp* was grown under one of the following conditions:

3.1 In the growth medium described in 1.1 above in a fermentor at room temperature under artificial lighting which altered in the range of between dim light (from about 1 W/m^2) to bright light (above about 10 W/m^2), typically under light of above 10 W/m^2 in the range of between 10 W/m^2 to about 200 W/m^2.

3.2 In outside pools at a temperature of between about 10°C to about 40°C under light conditions in the range of between 80% shade to full sunlight, typically between 40% shade to full sunlight.

4. Carotenoids extraction method

[0039] Phytoene and phytofluene were produced by inhibition of β-carotene synthesis in the algae *Dunaliella sp*. By the 4-chloro inhibitor at a concentration of 0.07 μM to 1.0 μM.

[0040] The algae were collected after four to six days of growth of the algae either outdoors or indoors as described above and the cell pellet was extracted with ethanol:hexane (1:2) v/v. Ethanol was first added to the cell pellet at algae: ethanol ratio of at least 1:10. At this stage basic hydrolysis of ester bonds is performed by the addition of 0.5M NaOH with stirring for at least 30 min. Ethanol:hexane phase separation is achieved by the addition of NaCl at adequate quantity. The hexane fraction was analyzed spectrophotometrically.

[0041] Hexane was dried under vacuum and the carotenoids were re-dissolved in hexane or oil.

5. Spectrophotometric analysis

[0042] Absorption spectra of the phytoene and phytofluene extract was determined using Hewlett Packard 8452A Diode Array Spectrophotometer.

6. Stability of phytoene and phytofluene under aerobic conditions in UV lights and various incubation temperatures

[0043] Stability of the carotenoids in the various temperatures was determined by long-term incubation of phytoene

and phytofluene in several types of oils and solvents at 4°C, 23°C, 30°C and 60°C. The amount of phytoene and phytofluene left was determined spectrophotometrically. The percent of phytoene and phytofluene left was calculated as follows:

$$\frac{\text{Phytoene and phytofluene after treatment (mg/ml)}}{\text{Phytoene and phytofluene before treatment (mg/ml)}} * 100 = \%\ \text{PH and PF left}$$

Stability under UV lights (254 nm, 365 nm, 254 + 365 nm), for 30 mins. at 300/310 μw/cm^2 was determined.

7. Stability of phytoene and phytofluene under aerobic conditions in visible light

[0044] Stability of phytoene and phytofluene under visible light was measured under sunlight (full sun summer day in Rehovot, Israel, at noon) filtered through "hot mirror" (Andover Corporation, Salem, NH, transmitance 400-650nm) filter. Samples were dissolved in ethanol, exposed to light for 30-150 min and then extracted in hexane after basic hydrolysis (as in extraction method above). Phytoene, phytofluene and beta-carotene amounts were measured spectrophotometrically, and percentage lefts were calculated as in section 5 above.

8. Anti-free radical activity

8.a Quenching of hydroxyl radicals:

[0045] The ability of the compound to quench the activity of hydroxyl radicals (°OH) was measured by Electron Paramagnetic Resonance (EPR), by comparing the signal intensity with and without the compound. Hydroxyl radicals were generated by decomposition of hydrogen peroxide by iron [($FeSO_4$) Fenton reaction]. The °OH is trapped by the DMPO to provide a DMPO-OH adduct, which presents a characteristic signal in EPR. The compound, composed of phytoene and phytofluene at a ratio of 6.66:1 respectively, was added to the system in three final dilutions of 1/20, 1/50 and 1/200.

[0046] Since various substances can reduce the signal, not by trapping of the °OH but by addition to the DMPO-OH adduct, it is necessary to also evaluate the signal when the product is added after the Fenton reaction (post-addition). The trapping capacity of the hydroxyl radical is evaluated theoretically by the difference between the value of the EPR signal corresponding to pre-addition of the product and the value of the signal resulting from post-addition.

8.b DNA protection by anti-oxidation effects:

[0047] DNA protection by the anti-oxidation effect of the compound was examined based on the method of Salles *et al*. (*Supra*). In the test the protective effect of the compound against Reactive Oxygen Species (ROS) generated oxidative DNA damage was measured, by quantifying the inhibition of DNA lesions formation using plasmid DNA target.

II. RESULTS

Example 1 Phytoene and phytofluene production

[0048] Phytoene and phytofluene were produced from the algae *Dunaliella sp*. by growing the algae in the presence of 0.1-0.5 μg of the β-carotene biosynthesis 4-chloro inhibitor as described above. The cultures were then extracted and the extract was dried by evaporation and re-dissolved in hexane or oil. No residues of the 4-chloro inhibitor were detected in the extracted carotenoids.

[0049] As seen in Fig. 1, the main absorbance peak of phytoene was found to be at 286 nm (UVB) and as seen in Fig. 2, the main absorbance peak of phytofluene was found to be at 348 nm (UVA). As expected, the main absorbance peak for β-carotene was at 450 nm. β-Carotene was reduced due to the synthesis inhibition while phytoene and phytofluene increased.

Example 2 Example for the stability of the extracted phytoene and phytofluene in UV lights

[0050] The stability of the phytoene and phytofluene extracts obtained as explained in Example 1 above was determined by spectrophotometric analysis. Phytoene and phytofluene were dissolved in hexane or oil. The absorbance of the preparation is determined in 220-600 nm and the amount of each of the above carotenoids in each sample is calculated to determine the percent of the carotenoids measured after irradiation of the extract divided by the amount of the same carotenoid which was measured in the extract before exposing it to irradiation (see Materials and Methods

5 above). The stability of the phytoene and phytofluene was measured by the exposure of the extract both to UVA light (365 nm) and UVB light (254 nm) irradiation as well as to a combination of UVA and UVB irradiation.

[0051] As seen in Table 1 below, phytoene and phytofluene dissolved in oil or hexane were very stable after exposure to UVA, UVB or UVA+B irradiation.

Table 1

PH and PF stability in UV irradiation (30 minutes exposure)				
	Darkness	**254 nm**	**365 nm**	**254nm+365nm**
	PH and PF (% left)[1]	PH and PF (% left)	PH and PF (% left)	PH and PF (% left)
PH left[1] in hexane	100	92.2	98.7	92.4
PF left[1] in hexane	100	92.6	101.5	99.0
PH left[1] in oil	100	100	100	101
PF left[1] in oil	100	90	88	98

[1] Percent of PH or PF left is the amount of material (mg/ml) measured after irradiation divided by the amount of material measured before treatment multiply by 100.

[0052] The stability of phytoene and phytofluene under different temperature conditions (4°C, 23°C, 30°C and 60°C) in hexane or in other various commercially used oils was also measured.

[0053] The measurements were carried out over a period of four months.

[0054] Phytoene and phytofluene were found to be stable in the entire measured temperature range with no substantial effect of the kind of oil in which the carotenoids were dissolved.

Example 3 Stability of the extracted phytoene and phytofluene in visible light

[0055] Stability of phytoene and phytofluene was compared to that of beta-carotene stability (as explained in materials and methods) under visible light. The absorbance of the preparation is determined in 220-600 nm wavelength and the amount of each of the above carotenoids in each sample is calculated to determine the percent of the carotenoids measured after irradation of the extract compared to measurements prior to irradiant (see example 2 above).

[0056] As shown in table 2, exposure to visible high intensity light cause significant higher degradation of beta-carotene compared to phytoene and phytofluene.

Table 2

PH and PF stability in visible light (15-150 min exposure)			
Time of exposure (min)	Phytoene (% left)	Phytofluene (% left)	Beta-carotene (% left)
0	100	100	100
30	54.46	66.13	25.00
90	20.33	50.42	11.36
120	15.70	41.33	9.47
150	13.64	33.06	5.68

Example 4 Anti free radical activity

4.a Quenching of hydroxyl radicals:

[0057] Quenching of hydroxyl radicals was measured by EPR as explained in materials and methods. The results obtained (Table 3) indicate a dose-dependent trapping activity of the hydroxyl radical by the product.

Table 3

Product final concentration (mg/ml)	Intensity of EPR signal (arbitrary units)	Intensity of the EPR signal after post-addition (arbitrary units)	% of hydroxyl radicals trapping
0	$2.82x10^6 \pm 0.07$		
0.001	$0.34x10^6 \pm 0.04$	$2.19x10^6 \pm 0.13$	66
0.0004	$0.58x10^6 \pm 0.02$	$1.94x10^6 - \pm 0.05$	48
0.0001	$1.26x10^6 \pm 0.10$	$2.19x10^6 \pm 0.13$	24

[0058] The product was composed of phytoene and phytofluene at a ratio of 6.66:1 respectively at an initial concentration of phytoene 0.02 mg/ml and phytofluene 0.003 mg/ml.

[0059] The average value of each result corresponds to three measurements.

4.b Anti oxidation effect:

[0060] DNA protection by the anti oxidation effect of the phytoene and phytofluene extracts was determined based on the Salles method (*Supra*) as explained in Materials and Methods 7.b above.

[0061] As seen in Table 4 below, phytoene and phytofluene obtained as described above were capable of protecting against hydroxyl radicals.

Table 4

PH and PF anti oxidation activity					
Compound	Concentration µg/ml	% of inhibition in the presence of ROS	% of non specific inhibition	% of specific inhibition	Concentration giving 50% of activity
Phytoene and phytofluene	140	86	24	62	11.2 µg/ml
	14	74	19	55	
	1.4	6	7	1	
	0.14	1	1	0	
Positive control	1000	88	9	79	80 µg/ml
	100	69	11	58	
	10	5	0	5	
	1	4	0	4	

Example 5 Protective effects on DNA by phytoene and phytofluene

Genotoxic effect

[0062] The genotoxicity is measured as the induction of DNA repair synthesis activity and is expressed by the ratio (R): while R≤ 2 indicate no toxic effect

$$R = \frac{\text{Relative Light Unit of the sample}}{\text{Relative Light Unit of the solvent alone}}$$

[0063] As seen in Table 5 below, phytoene and phytofluene are not genotoxic.

[0064] The R parameter is significantly less than 2 (protective effect) and much smaller in comparison with the positive control, which is a known genotoxic compound.

Table 5

Genotoxic effect of phytoene and phytofluene		
Compound	Concentration (μg/ml)	Genotoxicity (Ratio)*
Phytoene and phytofluene	140	0.92
	14	1.11
	1.4	1.18
	0.14	0.05
MMS-(positive control)	10 mM	6.26
	2 mM	2.44

* See above

Example 6

[0065] The following are examples of compositions that may be used in accordance with the invention:

A— *Emulsified gel of O/W (topical route):*

- Carbopol 981 (marketed by Goodrich) 0.6 g
- Ethyl alcohol 15 g
- Volatile silicone oil 3 g
- Purcellin oil 7 g
- Preserving agent 0.3 g
- Perfume 0.4 g
- Triethanolamine 0.2 g
- Phytoene 0.01 g
- Phytofluene 0.001 g
- Demineralized water.qs 100 g

B - *Anhydrous gel* (topical route):

- Propylene glycol 25 g
- Hydroxyethyl cellulose 0.8 g
- Polyethylene glycol 12 g
- Phytoene 1 g
- Phytofluene 1 g
- Absolute ethanol.qs 100 g

C- Emulsion of O/W type (topical route):

- Liquid paraffin 6 g
- Liquid lanolin 3 g
- Arlacel 165 (marketed by Atlas) 6 g
- Tween 60 (marketed by Atlas) 2 g
- Cetyl alcohol 1.2 g
- Stearic acid 2.5 g
- Volatile silicone oil 10 g
- Triethanolamine 0.1 g
- Preserving agent 0.3 g
- Antioxidants 0.3 g
- Phytoene 0.3 g
- Phytofluene 0.2 g
- Demineralized water.qs 100 g

D — *Cream containing liposomes* (topical route):

- Sunflower oil 35 g
- Cetyl alcohol 4 g
- B-sitosterol 4 g
- Dicetyl phosphate 0.5 g
- Preserving agent 0.3 g
- Parfume 0.6 g
- Carbopol 981 (marketed by Goodrich) 0.2 g
- Triethanolamine 0.2 g
- Sphingosine 005 g
- Phytoene 0.00001 g
- Phytofluene 0.000001 g
- Demineralized water.qs 100 g

E - *Per os composition*:

- Talc 5 g
- Aerosil 200 5 g
- Stearate de Zn 5 g
- Phytoene 0.0000015 g
- Phytofluene 0.000001 g
- Lactose qs 400 g

F — *Emulsion W.O* (topical route):

- Protegin (marketed by Goldschmidt) 19 g
- Glycerine 3 g
- Vaseline oil 8 g
- Phytofluene 0.5 g
- Phytoene 0.5 g
- Sulfate de Mg 0.5 g
- Perfume 0.8 g
- Preserving agent 0.2 g
- Water.qs 100 g

Claims

1. A composition active in prevention of oxidation or UV light exposure damage, **characterized in that**:

   - it comprises, as an active ingredient, a combination of phytoene and phytofluene;
   - it is essentially colorless;
   - it has an absorbancy spectrum at the UV wave range.

2. A composition according to Claim 1, being a topical skin preparation for protecting the skin against environmental hazards and comprising phytoene and phytofluene in an effective amount such that, in combination, these carotenoids exert an oxidation-protecting and UV-protecting effect on the skin.

3. A composition according to Claim 2, being a cosmetic or pharmaceutical composition.

4. A composition according to Claim 1, being a food additive.

5. A composition according to any of the previous claims, wherein the weight ratio between the phytoene and phytofluene in the composition is in the range of 200:1 to 1:200, respectively.

6. A composition in accordance with Claim 5, wherein the weight ratio between phytoene and phytofluene is 50:1 to 1:50, respectively.

7. A composition in accordance with Claim 6, wherein the weight ratio between phytoene and phytofluene in the composition is in the range of between 10:1 to 1:10, respectively.

8. A composition according to any of the previous claims, comprising a hydrophobic carrier.

9. A composition according to any one of the previous claims further comprising zetacarotene.

10. A method for preparing a composition comprising at least a combination of phytoene and phytofluene, comprising the steps of:

(i) incubating a carotenoid producing organism in a growth culture under broad spectrum light having an intensity of above 10 W/m^2 in the presence of one or more carotenoid synthesis inhibitors;
(ii) growing said carotenoid producing organisms until the level of at least one of phytoene or phytofluene is between 1 mg/l culture to 50 mg/l culture;
(iii) separating said organisms from the culture, and
(iv) preparing a preparation comprising a combination of phytoene and phytofluene or both from said separated organism.

11. A method according to Claim 10, wherein said light has an intensity in the range of between 20 W/m^2 to 200 W/m^2.

12. A method for preparing a composition comprising at least a combination of phytoene or phytofluene, comprising the steps of:

(i) Incubating a carotenoid producing organism in a growth culture under sunlight at an extent between 80% shading to full sunlight in the presence of one or more carotenoid synthesis inhibitors;
(ii) growing said carotenoid producing organisms until the level of at least one of phytoene or phytofluene is between 1 mg/l culture to 50 mg/l culture;
(iii) separating said organisms from the culture, and
(iv) preparing a preparation comprising a combination of phytoene and phytofluene or both from said separated organism.

13. A method according to Claim 12, wherein the extent of sunlight is between 80% shading to full sunlight.

14. A method according to Claim 12, wherein said organisms are grown under full sunlight.

15. A method according to any of Claims 10-14, wherein said preparation is an extract.

16. A method according to Claim 15, wherein said extract is an ethanol:hexane extract.

17. A method according to any of Claims 10-16, wherein said carotenoid producing organism is a micro algae.

18. A method according to Claim 17, wherein said micro algae is *Dunalielle sp*.

19. A method according to any of Claims 10-18, wherein said carotenoid synthesis inhibitor is 4-chloro-5(methylamino)-2-(3-(trifluoromethyl)phenyl)-3(2H)-pyridazinone.

20. A method according to any of Claims 15-19, wherein said extract comprises no traces of said inhibitor.

21. A method in accordance with any of Claims 14-20, further comprising the addition of charcoal to said carotenoid preparation during its preparation.

22. A composition in accordance with any of Claims 1-9, wherein said phytoene and phytofluene are prepared by chemical synthesis.

23. A composition in accordance with any of Claims 1-9, wherein said phytoene and phytofluene are prepared by recombinant methods.

Patentansprüche

1. Eine Zubereitung, die bei der Prävention von Oxidations- oder UV-Licht-Einwirkungs-Schäden wirksam ist, **dadurch gekennzeichnet, dass**

   - sie als Wirkstoff eine Kombination von Phytoen und Phytofluen umfasst,
   - sie im Wesentlichen farblos ist,
   - sie ein Absorptionsspektrum im UV-Wellenlängen-Bereich aufweist.

2. Eine Zubereitung gemäß Anspruch 1, welche ein topisches Haut-Präparat zum Schutz der Haut gegen Umweltrisiken ist und Phytoen und Phytofluen in einer wirksamen Menge enthält, so dass diese Carotinoide in Kombination eine Oxidationsschutz- und UV-Schutz-Wirkung auf der Haut ausüben.

3. Eine Zubereitung gemäß Anspruch 2, welche eine kosmetische oder pharmazeutische Zubereitung ist.

4. Eine Zubereitung gemäß Anspruch 1, welche ein Lebensmittelzusatz ist.

5. Eine Zubereitung gemäß einem der vorhergehenden Ansprüche, worin das Gewichtsverhältnis zwischen Phytoen und Phytofluen in der Zubereitung im Bereich von 200:1 bis 1:200 liegt.

6. Eine Zubereitung gemäß Anspruch 5, worin das Gewichtsverhältnis zwischen Phytoen und Phytofluen in der Zubereitung bei 50:1 bis 1:50 liegt.

7. Eine Zubereitung gemäß Anspruch 6, worin das Gewichtsverhältnis zwischen Phytoen und Phytofluen in der Zubereitung im Bereich von 10:1 bis 1:10 liegt.

8. Eine Zubereitung gemäß einem der vorhergehenden Ansprüche, die einen hydrophoben Träger umfasst.

9. Eine Zubereitung gemäß einem der vorhergehenden Ansprüche, die zusätzlich Zeta-Carotin umfasst.

10. Ein Verfahren zur Herstellung einer Zubereitung, die wenigstens eine Kombination von Phytoen und Phytofluen umfasst, umfassend die Schritte:

    (i) Inkubieren eines Carotinoid-produzierenden Organismus in einer Wachstumskultur unter Breitspektrum-Licht, das eine Intensität über 10 W/m^2 aufweist, in Gegenwart von einem oder mehreren Carotinoid-Synthese-Inhibitoren;

    (ii) Züchten dieser Carotinoid-produzierenden Organismen, bis der Spiegel von wenigstens Phytoen oder Phytofluen zwischen 1 mg/l Kultur bis 50 mg/l Kultur liegt;

    (iii) Abtrennen der Organismen von der Kultur; und

    (iv) Herstellen eines Präparats, das eine Kombination aus Phytoen und Phytofluen oder beide umfasst, aus dem abgetrennten Organismus.

11. Ein Verfahren gemäß Anspruch 10, worin das Licht eine Intensität im Bereich von zwischen 20 W/m^2 bis 200 W/m^2 aufweist.

12. Ein Verfahren zur Herstellung einer Zubereitung, die wenigstens eine Kombination von Phytoen und Phytofluen umfasst, umfassend die Schritte:

    (i) Inkubieren eines Carotinoid-produzierenden Organismus in einer Wachstumskultur unter Sonnenlicht in einem Bereich von 80 % Verschattung bis volles Sonnenlicht in Gegenwart von einem oder mehreren Carotinoid-Synthese-Inhibitoren;

    (ii) Züchten dieser Carotinoid-produzierenden Organismen, bis der Spiegel von wenigstens Phytoen oder Phytofluen zwischen 1 mg/l Kultur bis 50 mg/l Kultur liegt;

(iii) Abtrennen der Organismen von der Kultur; und

(iv) Herstellen eines Präparats, das eine Kombination aus Phytoen und Phytofluen oder beide umfasst, aus dem abgetrennten Organismus.

13. Ein Verfahren gemäß Anspruch 12, worin der Sonnenlicht-Bereich zwischen 80 % Verschattung bis volles Sonnenlicht liegt.

14. Ein Verfahren gemäß Anspruch 12, worin die Organismen unter vollem Sonnenlicht gezüchtet werden.

15. Ein Verfahren gemäß einem der Ansprüche 10 bis 14, worin das Präparat ein Extrakt ist.

16. Ein Verfahren gemäß Anspruch 15, worin das Extrakt ein Ethanol:Hexan-Extrakt ist.

17. Ein Verfahren gemäß einem der Ansprüche 10 bis 16, worin der Carotinoidproduzierende Organismus eine Mikro-Alge ist.

18. Ein Verfahren gemäß Anspruch 17, worin die Mikro-Alge *Dunalielle sp.* ist.

19. Ein Verfahren gemäß einem der Ansprüche 10 bis 18, worin der Carotinoid-Synthese-Inhibitor 4-Chlor-5(methylamino)-2-(3-(trifluormethyl)phenyl)-3(2H)-pyridazinon ist.

20. Ein Verfahren gemäß einem der Ansprüche 15 bis 19, worin das Extrakt keine Spuren des Inhibitors umfasst.

21. Ein Verfahren gemäß einem der Ansprüche 14 bis 20, das zusätzlich die Zugabe von Kohle zum Carotinoid-Präparat während dessen Herstellung umfasst.

22. Eine Zubereitung gemäß einem der Ansprüche 1 bis 9, worin das Phytoen und das Phytofluen durch chemische Synthese hergestellt werden.

23. Eine Zubereitung gemäß einem der Ansprüche 1 bis 9, worin das Phytoen und das Phytofluen durch rekombinante Verfahren hergestellt werden.

Revendications

1. Composition active pour la prévention de l'oxydation ou d'une lésion suite à une exposition à une lumière UV, **caractérisée en ce que** :

- elle comprend, en tant qu'ingrédient actif, une combinaison de phytoène et de phytofluène ;
- elle est essentiellement incolore ;
- elle présente un spectre d'absorbance dans la gamme des ondes UV.

2. Composition selon la revendication 1, qui est une préparation locale pour la peau pour protéger la peau contre les risques environnementaux et comprenant un phytoène et un phytofluène en une quantité efficace de telle sorte que, en combinaison, ces caroténoïdes exercent un effet de protection contre l'oxydation et de protection contre les UV sur la peau.

3. Composition selon la revendication 2, qui est une composition cosmétique ou pharmaceutique.

4. Composition selon la revendication 1, qui est un additif alimentaire.

5. Composition selon l'une quelconque des revendications précédentes, dans laquelle le rapport pondéral du phytoène au phytofluène dans la composition est dans la gamme de 200:1 à 1:200, respectivement.

6. Composition selon la revendication 5, dans laquelle le rapport pondéral du phytoène au phytofluène est de 50:1 à 1:50, respectivement.

7. Composition selon la revendication 6, dans laquelle le rapport pondéral du phytoène au phytofluène dans la composition est dans la gamme de 10:1 à 1:10, respectivement.

8. Composition selon l'une quelconque des revendications précédentes, comprenant un véhicule hydrophobe.

9. Composition selon l'une quelconque des revendications précédentes, comprenant en outre un zéta-carotène.

10. Procédé de préparation d'une composition comprenant au moins une combinaison de phytoène et de phytofluène, comprenant les étapes consistant à :

(i) incuber un organisme produisant un caroténoïde dans une culture de croissance sous une lumière à large spectre ayant une intensité supérieure à 10 W/m^2 en présence d'un ou de plusieurs inhibiteurs de synthèse de caroténoïde ;
(ii) cultiver lesdits organismes produisant un caroténoïde jusqu'à ce que la teneur d'au moins un élément parmi le phytoène ou le phytofluène soit compris entre 1 mg/l de culture et 50 mg/l de culture ;
(iii) séparer lesdits organismes de la culture ; et
(iv) préparer une préparation comprenant une combinaison de phytoène et de phytofluène ou les deux à partir dudit organisme séparé.

11. Procédé selon la revendication 10, dans lequel ladite lumière a une intensité dans la gamme comprise entre 20 W/m^2 et 200 W/m^2.

12. Procédé de préparation d'une composition comprenant au moins une combinaison de phytoène et de phytofluène, comprenant les étapes consistant à :

(i) incuber un organisme produisant un caroténoïde dans une culture de croissance sous une lumière solaire à un niveau compris entre 80 % d'ombre et une lumière solaire intense en présence d'un ou de plusieurs inhibiteurs de synthèse de caroténoïde ;
(ii) cultiver lesdits organismes produisant un caroténoïde jusqu'à ce que la teneur d'au moins un élément parmi le phytoène ou le phytofluène soit compris entre 1 mg/l de culture à 50 mg/l de culture ;
(iii) séparer lesdits organismes de la culture ; et
(iv) préparer une préparation comprenant une combinaison de phytoène et de phytofluène ou les deux à partir dudit organisme séparé.

13. Procédé selon la revendication 12, dans lequel le niveau de lumière solaire est compris entre 80 % d'ombre et la lumière solaire intense.

14. Procédé selon la revendication 12, dans lequel lesdits organismes sont cultivés sous une lumière solaire intense.

15. Procédé selon l'une quelconque des revendications 10 à 14, dans lequel ladite préparation est un extrait.

16. Procédé selon la revendication 15, dans lequel ledit extrait est un extrait dans un mélange éthanol:hexane

17. Procédé selon l'une quelconque des revendications 10 à 16, dans lequel ledit organisme produisant un caroténoïde est une micro-algue.

18. Procédé selon la revendication 17, dans lequel ladite micro-algue est *Dunalielle sp*.

19. Procédé selon l'une quelconque des revendications 10 à 18, dans lequel ledit inhibiteur de synthèse de caroténoïde est la 4-chloro-5-(méthylamino)-2-(3-trifluorométhyl)phényl)-3(2H)-pyridazinone.

20. Procédé selon l'une quelconque des revendications 15 à 19, dans lequel ledit extrait ne contient aucune trace dudit inhibiteur.

21. Procédé selon l'une quelconque des revendications 14 à 20, comprenant en outre l'addition de charbon à ladite préparation de caroténoïde pendant sa préparation.

22. Composition selon l'une quelconque des revendications 1 à 9, dans laquelle ledit phytoène et ledit phytofluène

sont préparés par une synthèse chimique.

23. Composition selon l'une quelconque des revendications 1 à 9, dans laquelle ledit phytoène et ledit phytofluène sont préparés par des procédés de recombinaison.

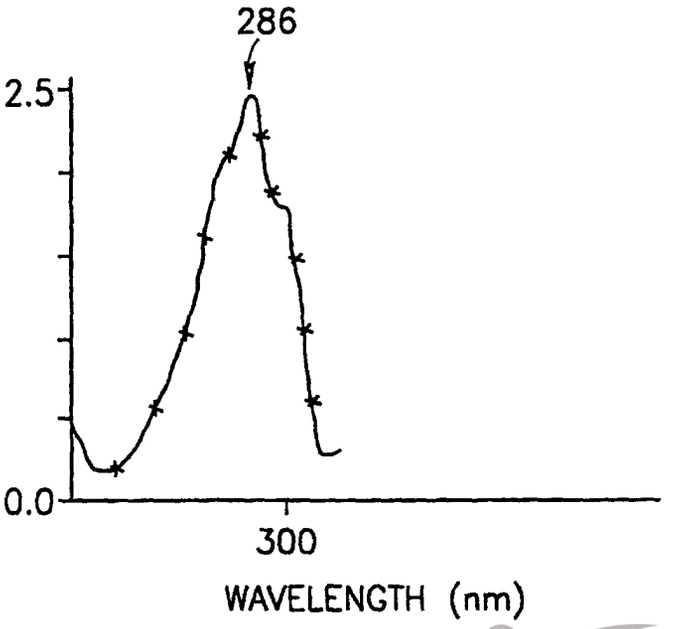

FIG.1

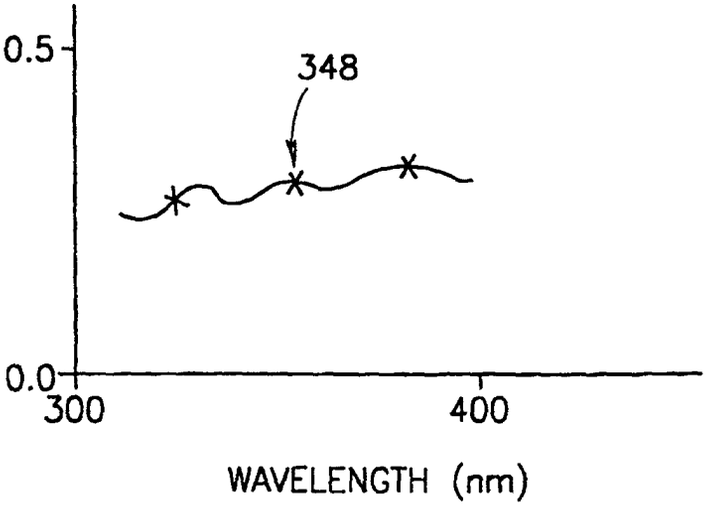

FIG.2