INTRODUCTION

Free radical-mediated lipid peroxidation has been proposed to be critically involved in several desease states including cancer, rheumatoid arthritis, drug associated toxicity, and postischemic reoxygenation injury, as well as in the degenerative processes associated with aging (1-3).
Higher organisms place a considerable emphasis on defense against oxidative damage. The defense mechanisms involve both enzyme and small molecules. The former are represented by superoxide dismutase, catalase and glutathione peroxidase. The latter include vitamin E and β-carotene, which act as free radical scavengers in membranes, and vitamin C, uric acid and albumin bound bilirubin, which perform this function in the acqueous phase (4). Protein sulfhydryl groups and glutathione have also been suggested to play a pivotal role in cellular antioxidant defenses (5). Skin is an highly differentiated and complex organizated structure, and with age or in particular conditions become more vulnerable to free radical induced damage (i. e . hypersensitivity, degenerative alterations, photocarcinogenesis) (6-7). In view of the hypothesis that antioxidants may be particularly indicated to protect skin from oxidative stress, we investigated lipoperoxidation in skin lipids after topical treatment with different fractions of lemon oil and compared them to those obtained with vitamin E.

MATERIALS AND METHODS

Treatment and Sampling

The study was limited to 10 adult male volunteers. The age of the subjects ranged from 18-52 years old. The mean age in the group was 33 ± 11. The subjects were requested to avoid the use of hair lotions or other oil containing ointments during the duration of the experimental period.
The experimental procedure was designed to evaluate the effectiveness of the lemon oil extract (L.O. -22) as compared to vitamin E in preventing lipid peroxidation of skin surface lipids. The study was carried out in the Dermatology Clinic at the University Hospital. The 10 volunteers were divided randomly into two groups (A and B) of 5 individuals. During the first week, group A was asked to apply vitamin E on days 1, 3, and 5 whereas the group B was asked to use L.O.-22 on days 1, 3 and 5. At the end of the first week of treatment, the volunteers were asked to return to the Dermatology Clinic one week later. Upon re-entry, the groups were subjected to a cross over trial. Group A was instructed to use L.O.-22 on days 1, 3 and 5 whereas group B was asked to use vitamin E on the corresponding days.

Experimental procedure

At the beginning of the experiment a sample of skin surface lipid was obtained from the forehead of each individual. The surface lipids were obtained by swabbing an area of the forehead (3 cm x 3 cm) with a cotton swab. The procedure was standardized such that the area was swabbed three times horizzontally and three times vertically for each individual. The two groups where then asked to wash the forehead thoroughly with neutral soap and then group A was given a vial containing 3 ml of 5% alpha-tocopherol solubilized in 20% ethanol to apply freely on the forehead. The application of vitamin E or L.0.-22 was repeated on days 3 and 5. Samples of skin lipids obtained by the swabbing technique were taken at 1, 2, 4, 6, 8 and 24 hours after application of vitamin E or L.0.-22. This experiment was carried out on days 1, 3 and 5.

Extraction of lipids

The cotton swabs were extracted for their lipid content with 3 ml of chloroform:methanol mixture (1:2.5). The extraction procedure was al-

lowed to continue at room temperature for 2 hours in the presence of heneicosanoic acid (10 µg) as internal standard. The cotton was removed and re-extracted with fresh chloroform:methanol solution (1 ml). The extraction solutions were combined and 1% NaCl in 0.01 M HCl was added to the mixture and centrifuged. The chloroform layer from both extractions was pooled and washed with 3 ml methanol:water (1:1). The solution was centrifuged and after phase separation, the chloroform layer was recovered and evaporated to dryness under a stream of nitrogen gas in the dark. The dried lipids were dissolved in 3 ml chloroform:methanol solution (2:1) and the solution was stored at 20°C in the dark until analysis of lipid content and lipid peroxidation studies.

Peroxidative stress of skin surface lipids

The sensitivity of skin surface lipids extracted from individuals in the two experimental groups was evaluated by measuring the susceptibility of the lipids to oxidative stress in the presence of ferrous ions-EDTA complex (0.2 M) at pH 7.5. Oxidative damage to lipids generally leads to the formation of malonaldehyde (MDA) which has been investigated extensively as a marker for lipid peroxidation processes in vitro and in vivo.

Assay for MDA

MDA was measured using a micromethod modified from Slater and Sawyer (8): to 0.5 ml of the mixture of reaction were added 0.5 ml of 20% (w/v) thrichloroacetic acid; after centrifugation, 0.9 ml of the snpernatant fraction was added to 1 ml of 67% thiobarbituric acid (Sigma) dissolved in 0.026 M Tris-HCl buffer pH 7.0. The samples were heated in boiliug water for 10 min. After cooling, the absorbance was determined at 532 nm on a Perkin Elmer mod.559 spectrophotometer. Extraction blanks were prepared and treated in the same way as the experimental samples but an equal volume of buffer was substituted for reaction mixture. MDA was quantitated using MDA standard (Aldrich) and expressed in nanomoles per nanomoles of phosphate.

Assay for phosphorus

Phosphorus was measured using an ultramicro modification from Bartlett (9).Aliquots (1.5 ml) of lipid extract were combusted adding 0.3 ml of 10 N sulphuric acid and heating the mixture at 200 °C for 3 hours. Two drops of 30% hydrogen peroxide were added and the solution was heated for 1.5 hours more at 200 °C to complete the combustion and to decompose all the peroxide. After combustion, 0.65 ml of distilled water, 0.2 ml of 5% ammonium molybdate and 0.05 ml of the Fiske-Subbarow reagent were added, and the solution was heated for 7 minutes at 100°C. The optical density was read at 830 nm. Inorganic orthophosphate was used to prepare the standard curve.

Fatty acids analysis

Lipids contained in half of the extract were transesterified in 1 ml of 2% sulphuric acid in methanol:benzene (1:1 v/v) for 4 hours at 65°C. Methyl esters so obtained were brought to dryness under a gentle stream of nitrogen and resuspended in 2 ml of hexane plus 1 ml of methanol. The exane layer was then transferred in 3 ml vials, provided with teflon-lined screw caps, dried and resuspended in 100 µl of exane. Fatty acid analysis was carried out with a Carlo Erba gas- chromatograph (mod. Fractovap 4200). SE 30 (3% on 80/100 mesh Chromosorb WHP) was used as the stationary phase in 2 m x 2mm ID glass column with nitrogen as carrier gas. The temperature was programmed from 160°C to 260°C at a rate of 8°C/min. The detector and injector temperatures were set at 260 °C. Quantitative measurements and calculations were effe-

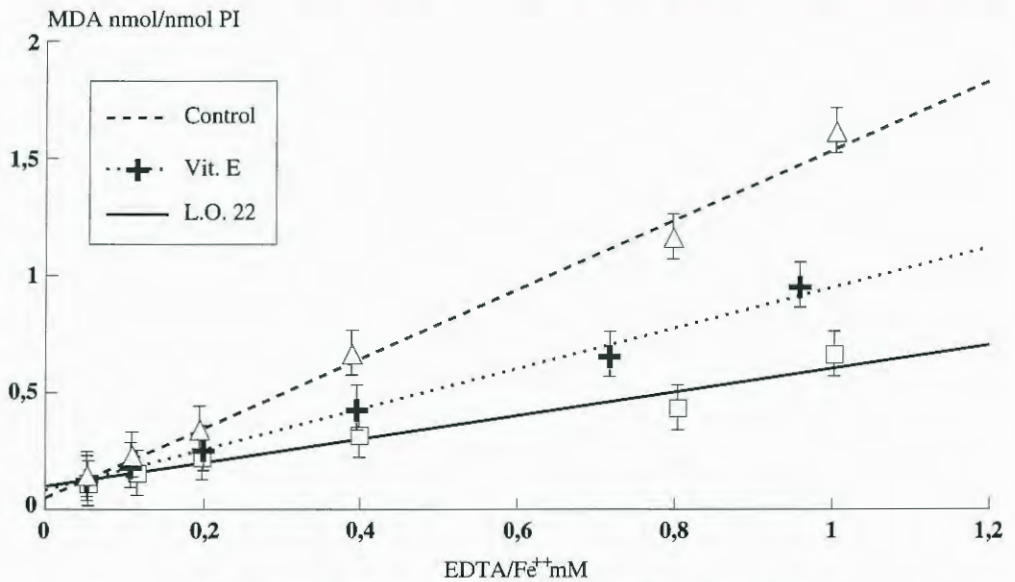
cted by comparing peak height ratios between acids and internal standard of samples with those of standard solutions of comparable concentrations. Results were expressed as µmoles of fatty acids per nmoles of inorganic phosphate. Student's t test was used for estimating the significance of difference between group means.

RESULTS

The antioxidant activity of vitamin E has been widely recognized over the past years and hence has provided a useful guideline for evaluating the antioxidant activity of other natural products. In our study, we have compared vitamin E to a natural antioxidant denominated L.0.-22. This product was fractionated from a lemon oil extract via steam distillation followed by column chromatography of the distillate on phasesorb and eluted with ethanol:water mixture (3:1). The chemical characteristics of this fraction suggest the presence of an isoprenoid - quinoid compound wich exhibits strong antioxidant activity in in vitro assay system as shown in figure 1. As can be seen, the addition of increasing concentration of Fe-EDTA complex to a lipid extract increased the production of malonaldehyde (MDA). However, this increase was significantly reduced when the incubation medium contained 5% vitamin E or when the incubation medium was made 5% with respect to L.0.-22. As can be seen from figure 1, at the same concentration of vitamin E and L.0.-22, the latter product was significantly more effective than the vitamin E compound in inhibiting lipid peroxidative formation of malonaldehyde. A

Figure 1

MDA PRODUCTION AS FUNCTION OF EDTA-Fe^{++} CONCENTRATION



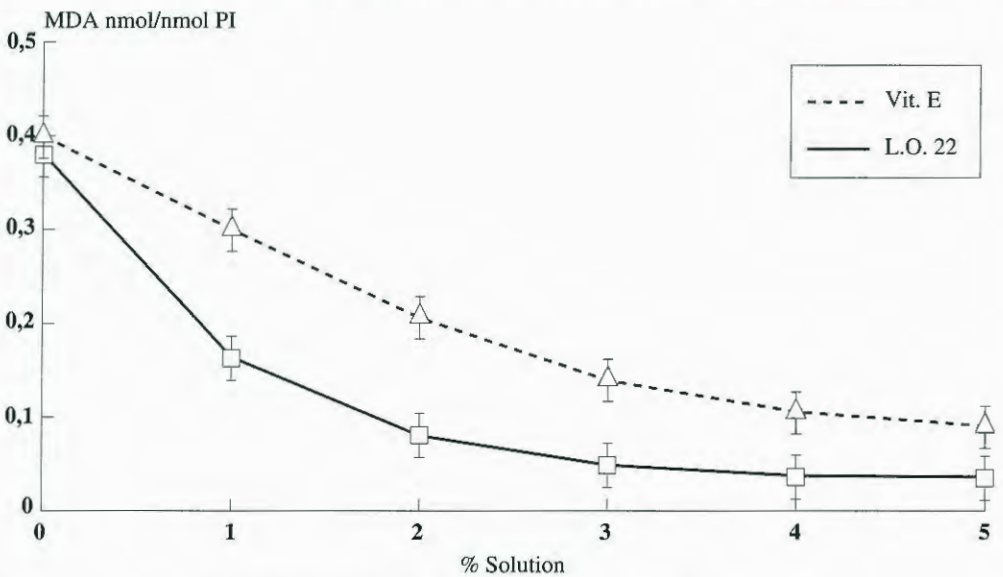
Results are the mean ± S.D. of 5 experiments.

dose dependent response was also performed for the in vitro assay system, figure 2. The data show that at concentration ranges of 0.01% to 1% the L.0.-22 antioxidant activity was approximately 30-50% higher as compared to a similar dose range of vitamin E. At higher concentration ranges (1%-5%), L.0.-22 inhibited the production of MDA by 15-20%. The differences in the dose-response activity of L.0.-22 suggests a possible aggregation phenomenon at higher concentrations and hence reduces the effective antioxidant activity of the product. In contrast, vitamin E did not display this dose dependent inhibition of MDA production.

The susceptibility of lipids toward peroxidative stress were evaluated using lipid sample collected in the study with male volunteers. As can be seen from figure 3, the L.0.-22 treated subjects, as compared to vitamin E group, consistently exhibited a higher resistence to lipid peroxidative stress reactions as shown by the decreased production of MDA. Similar results were obtained during the cross over trial of groups A and B, figure 4. This study demonstrated that treatment with L.0.-22 significantly inhibited peroxidative damages to skin lipids. In order to investigate further the mechanism of action of L.0.-22, we analyzed the various lipid classes in each sample of skin lipid and, as reported in figure 5, we found that peroxidative damage was decreased significantly for short chain fatty acids. Hence treatment with L.0.-22 protects against peroxidative damage of lipid fatty acids in general and in particular the short chain fatty acids.

Figure 2

MDA PRODUCTION IN THE PRESENCE OF ANTIOXIDANTS



Results are the mean ± S.D. of 5 experiments.

Figure 3

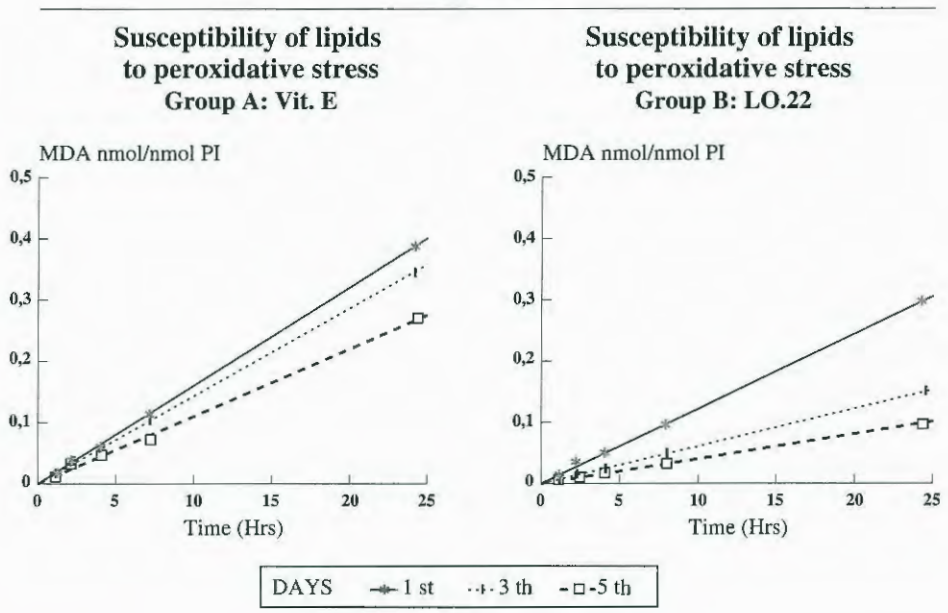
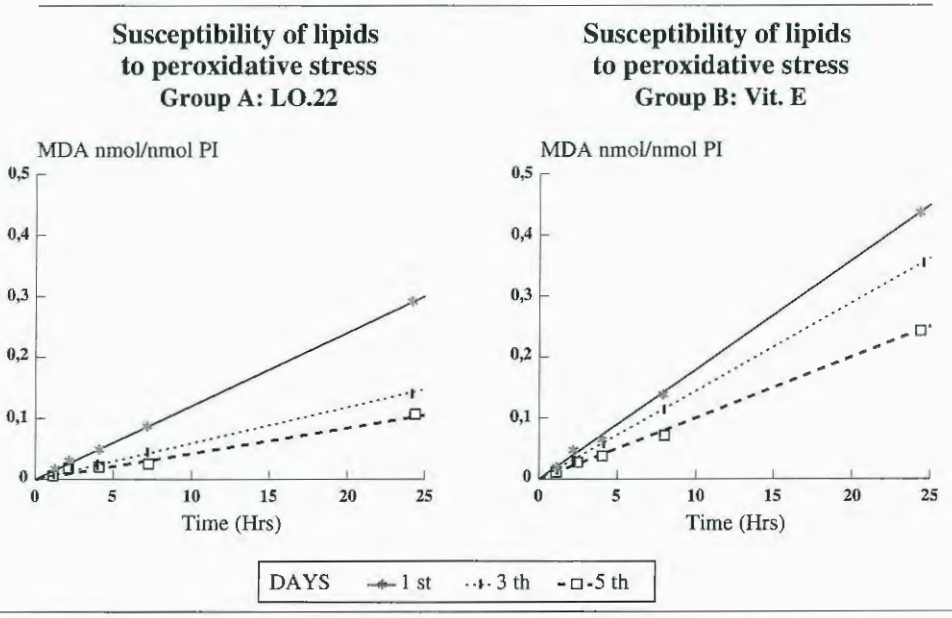


Figure 4



DISCUSSION

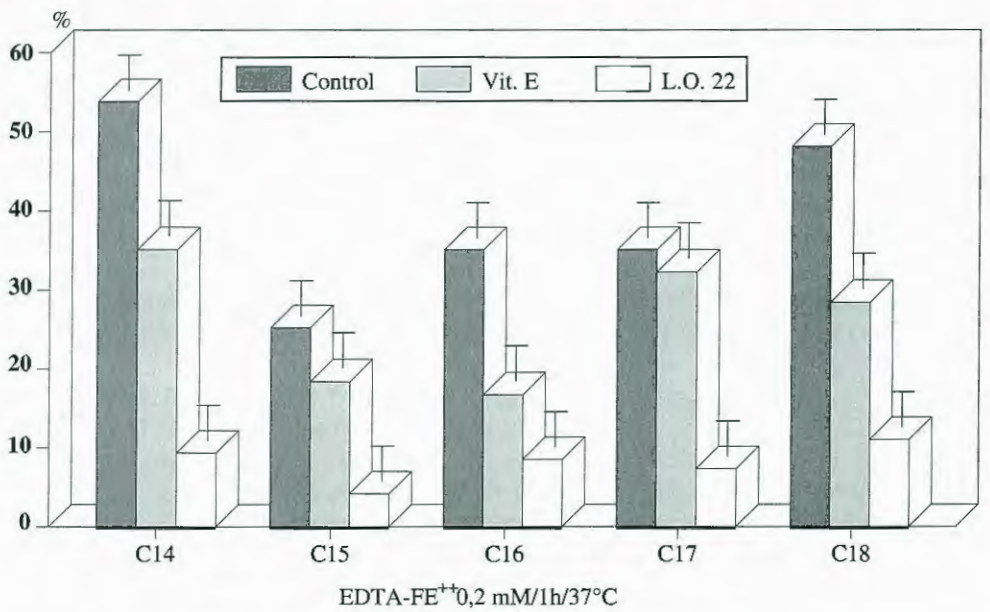
Damage to biological system caused by generation of active oxygen species is often referred to as "oxidative stress" (11). There is now growing evidence for the involvement of oxygen radicals and other oxygen-derived species as causative agents in aging and several human diseases. Skin is an highly differentiated and certainly complex organizational structure which, with age, becomes particularly vulnerable to a cohort of degenerative disorders. These disorders have been correlated not only to immunological changes - altered differentiation of lymphocytes, involution of thymus - but also to a free radical-mediated induction of hypersensitive and photocarcinogenetic processes (6-7). In the recent years, various experimental models have been developed in order to investigate the molecular mechanism by which active oxygen species cause cell and tissue damage and to find suitable treatments capable of preventing such damage.

In our study we employed a peroxidative system to mime pathogenic condition supposed to be involved in the aging of the skin. In this system, skin surface lipids of normal subjects are stressed in presence of EDTA-Fe, with production of malonaldehyde, one of the most studied by-products of cellular peroxidative damage. MDA formation linearly increases as a function of EDTA-Fe concentration.

Also, we studied lipoperoxidation through direct analysis of skin fatty acids, founding a strong correlation between decrease in the unsaturated/saturated fatty acid ratio (which is an in-

Figure 5

% PERIOXIDATION OF SKIN LIPIDS COLLECTED AT 24 HRS OF 5th DAY



Results are the mean ± S.D. of 5 experiments.

dex in the peroxidative breakdown of unsaturated fatty acids) and MDA formation in presence and in absence of different antioxidants (10). Topic treatment with vitamin E, or different fractions isolated from lemon oil extract, provided significant protection against oxidative stress. Among the compounds investigated, the fraction indicated as L.0.-22 exhibited the best protective effects against free radical induced injury, as compared to vitamin E. This study indicates that a fraction isolated from lemon oil extract, may be used to prevent lipoperoxidative changes of the skin, pointing out the importance of a natural antioxidant technology in the anti-aging treatment of the skin.

REFERENCES:

1. **Pryor W.A. (1977)** Free radicals in biology. The involvement of radical reactions in aging and carcinogenesis. In W.A. Pryor (Ed.), *Medicinal Chemistry*, **Vol. 5**, Elsevier Scientific Publications, New York, pp. 331-359.
2. **Halliwell B. (1989)** Free radicals, reactive oxygen species and human disease: A critical evaluation with special reference to the atherosclerosis. *Br. J. Exp. Pathol* **70**, 737-757.
3. **Halliwell B. and Gutteridge J.M.C. (1990)** The antioxidants of human extracellular fluids. *Arch Biochem Biophys* **280**, 1-8.
4. **Waguer D.D.M., Burton G.W., Ingold K.U., Barclay L.R.C., Locke S.J. (1987)** The relative contribution of vitamin E, urate, ascorbate and proteins to the total peroxyl trapping antioxidant activity of human blood plasma. *Biochim Biophys Acta* **924**,408- 419.
5. **Meister A. (1991)** Glutathione deficiency produced by inhibition of its synthesis and its reversal; Applications in research and therapy. *Pharmacol Ther* **51**, 155- 194.
6. **Athar M., Elmet C., Bickers D.R., Mukhtar H. (1989)** A novel mechanism for the generation of superoxide anions in hematoporphyrin derivative mediated cutaneous photosensitization. *J. Clin. Inv.* **83**,1137-1143.
7. **Giannetti A. (1986)** Immunological changes in aging skin. In P. Morganti, W. Montagna (Eds.), *Cosmetic Dermatology*, Vol. 1, International Ediemme, Rome, Italy, pp. 139-143.
8. **Slater T.F., Sawyer B.C. (1971)** The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions in vitro, *Biochem J.*, **123**, 805-814.
9. **Bartlett J.R. (1959)** Phosphorus assay in column chromatography, *J. Biol. Chem.*, **234**, 466-468.
10. **Calabrese V., Randazzo S.D., and Rizza V. (1991)** An in vivo biochemical system to assess the antioxidant, antiaging effects of cosmetic products. *J. Appl. Cosmetol.* **9**, 65-71.
11. **Sies H. (Ed.) (1985)** "Oxidative Stress". Academic Press, London