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RESEARCH ACTIVITY

under the agreement between

PHYTOLAB (Pharmaceutical, Cosmetic, Food Supplement, Technology and Analysis)
Science and Technology Center of the University of Florence

and

DOMUS OLEA TOSCANA, San Giuliano Terme (Pisa, Italy):

Production of vegetable extracts from Urtica dioica L. (Nettle leaves), Vitis vinifera L. (Grape leaves), Olea europaea L. (Olive leaves) and Helichrysum italicum (Roth) (whole plant) for cosmeceutical use.

CHARACTERIZATION AND EVALUATION OF THE ANTI-FREE RADICAL/ANTI-AGEING ACTIVITY OF DOMUS OLEA TOSCANA PRODUCTS.

INTRODUCTION

Domus Olea Toscana produces natural cosmetics, organic certified by ICEA under EU regulations. Their products are manufactured using local ingredients from Tuscany and possess the requirements to be highly concentrated in functional compounds effective against ageing.

The aim of the present research is to achieve plant extracts highly concentrated in antioxidants and anti-free radical molecules, to be used for the production of high performing anti-ageing cosmetics, commonly known as "Cosmeceuticals".

The prevention of skin aging is one of the most interesting research topic of modern cosmetology. The physiological process of ageing overlaps the premature ageing caused by extrinsic factors, such as UV radiation and pollutants. This process is caused by damage to the connective tissue, mainly due to oxidative reactions. The use of antioxidants is therefore an important strategy in the prevention and treatment of oxidative damage. Particular attention is currently dedicated to the natural antioxidants, including phenolic compounds, such as Oleuropein, Tyrosol and Hydroxytyrosol, which are mainly contained in Olive leaves.

Phenolic compounds are "chain-breaking" antioxidants, whose protective effect against

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the lipoperoxidative damage depends on the ability of the hydroxyl group to donate hydrogen ions. Their activity is related to the ability to interact with biological membranes. Oleuropein is an amphipathic molecule able to pass through cell membranes as Hydroxytyrosol. Hydroxytyrosol passes through cell membranes by a mechanism of passive diffusion and neutralizes the cytotoxic effects of free radicals.

Thanks to patented extraction technologies, PHYTOLAB is able to achieve plant extracts, which have a content of antioxidants and anti-free radicals from 5 to 10 times higher than the extracts commonly available on the market.

MATERIALS AND METHODS

Plant materials

All samples were provided by Domus Olea Toscana.

Extraction

To organic Olive, Grapevine, Nettle and Helichrysum plants, a sustainable extractive technology, using a pneumatic extractor (Timatic), was applied by heating at 80°C, in order to obtain aqueous extracts enriched in antioxidant compounds, then suitable for cosmetic applications. Extracts were characterized and quantified by HPLC/DAD/ESI-MS and, then, antiradical and antioxidant properties were evaluated for each cosmetic products.

HPLC/DAD and HPLC/MS analyses

HPLC/DAD analyses of flavonols, phenolic acids and minor polar compounds were carried out using an HP 1100L liquid chromatograph equipped with a DAD detector and managed by an HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). A four-step linear solvent gradient starting from 100% H₂O up to 100% CH₃CN was performed with a flow rate of 0.8 mL min⁻¹ during for 88-min period. The column was a Lichrosorb C18 250×4.60 mm, 5µm (LichroCART, Merck) operating at 26°C. The eluents were H₂O adjusted to pH 3.2 by HCOOH/CH₃CN. UV/Vis spectra were recorded in 190–600 nm range and chromatograms acquired at 240, 280, 330 and 350 nm.

HPLC/MS analyses were performed using an HP 1100L liquid chromatograph equipped with an HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer operating conditions were: gas temperature, 350°C; nitrogen flow rate, 10.0 L min⁻¹, nebulizer pressure 30 psi; quadrupole temperature, 30°C; and capillary voltage, 3500 V. The mass spectrometer operated in negative ionization mode at 80–120 eV.



Polyphenol Characterization and Quantification

The identity of polyphenols was ascertained using data from HPLC/DAD and HPLC/MS analyses by comparison and combination of their retention times, UV/Vis, and mass spectra with those of authentic standards. Quantification of individual polyphenolic compounds was directly performed by HPLC/DAD using a five-point regression curve with the available standards. Curves with an $R^2 \geq 0.998$ were considered. Calibration was performed at the wavelength of the maximum UV–Vis absorbance, applying the correction of molecular weight. In particular, secoiridoids as oleuropein were measured at 280 nm, elenolic acid derivatives at 240 nm, hydroxytyrosol and derivatives as tyrosol at 280 nm, verbascoside and other hydroxycinnamic derivatives as chlorogenic acid at 330 nm, and, finally, flavonoids were calibrated using the specific pure compounds at 350 nm.

Determination of antiradical activity

The antiradical activity was evaluated using the test of the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl), according to a previously reported procedure with slight modifications. In detail, the extracts were opportunely diluted and added, in a 1:1 amount, to an ethanolic solution of DPPH (0.025 mg mL^{-1}). Measurements were carried out at 517 nm with a spectrophotometer DAD 8453 (Agilent Technologies) at time 0 and every 2 min for the following 20 min.

Antiradical activity (AR%) was calculated through the relationship:

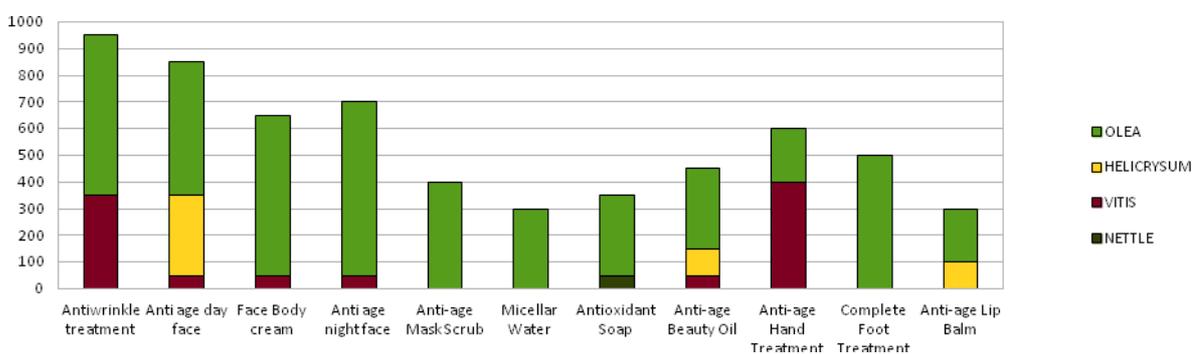
$$[\text{AR}\% = 100 (A_0 - A_{20})/A_0]$$

where A_0 and A_{20} were the absorbance of DPPH, respectively at time 0 and after 20 min from the addition of the diluted extract. The EC_{50} of the extracts was determined through the use of five-point linearized curves [$\text{AR}\% - \ln(\text{concentration in polyphenols})$], built determining AR% for five different dilutions of each extract, and then calculating the polyphenols concentration as mg of antioxidants per gram of cosmetic product that inhibits the DPPH activity to 50%, smaller this value more efficient is the product.



RESULTS AND DISCUSSIONS

Domus Olea Toscana anti-age products have been developed with the aim to achieve a high anti-free radical activity (AR%), greater than 60% for face and body creams (classified as high anti-radical activity in the pharmaceutical community and, therefore, anti-ageing).



The above chart shows the amount of polyphenols extracted from Olea, Grapevine, Nettle and Helichrysum (anti-oxidant/anti-radical) included in each reference (mg kg⁻¹ of product) in order to obtain the target anti-radical activity.

DPPH test has been performed in order to evaluate antioxidant and antiradical properties of Domus Olea Toscana products, since DPPH test is an internationally recognized *in vitro* test used to measure the anti-free radical activity of a product.

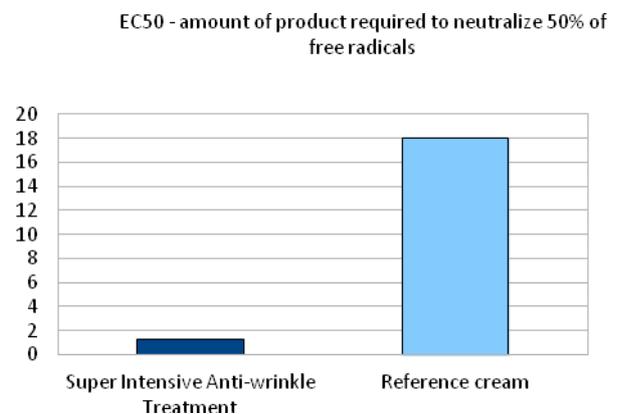
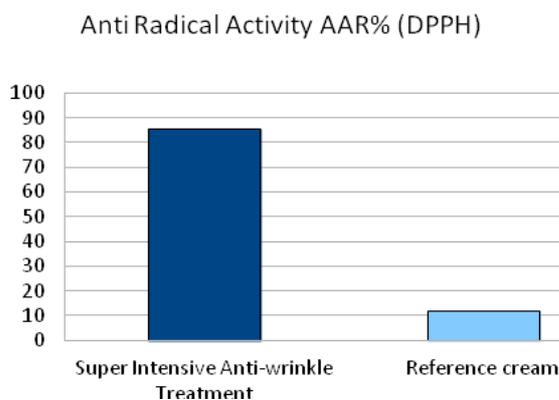
DPPH findings can be correlated with the biological activities that these active principles exert into our body, towards endogenous radicals, such as hydroxyl, superoxide and singlet oxygen, which are the main responsible of skin ageing.

Tests were carried out on Domus Olea Toscana products and on a reference cosmetic cream containing a set of antioxidants such as vitamin A, E, C, commonly available on the market.



TEST RESULTS ON MAIN PRODUCTS

Super-intensive Antiwrinkle Treatment

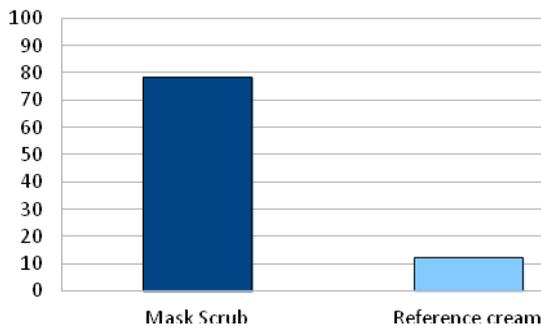


Test results show that the Super-Intensive Anti-wrinkle treatment has a very high anti-radical activity and that quantities much lower than the reference cream are sufficient to neutralize anti-free radical present in the skin. Among the products Domus Olea Toscana, it is the most effective and efficient, which justify the use of a small amount of product to have an immediate effect on high-risk areas of oxidation, inflammation and aging (i.e. wrinkles around the eyes and around lips).

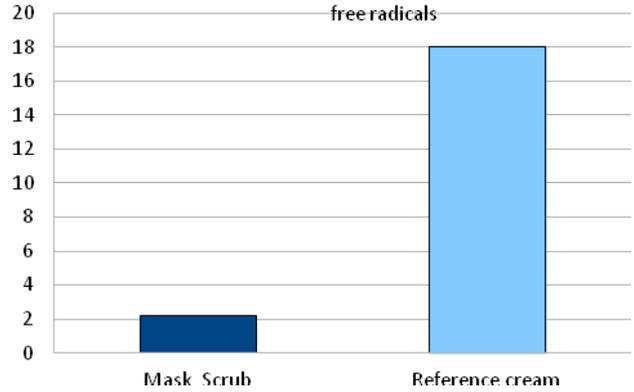


Anti-age Mask-Scrub Peeling

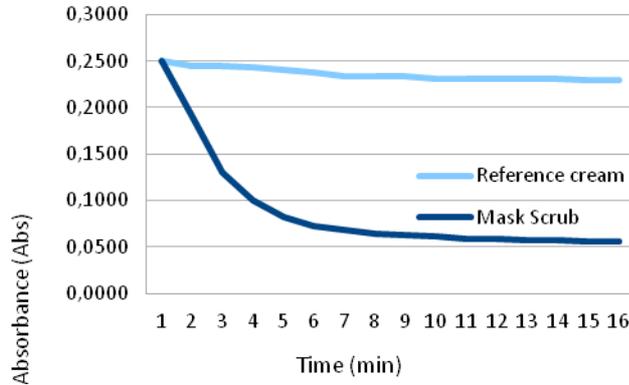
Anti Radical Activity (AAR)% (DPPH)



EC50 - amount of product required to neutralize 50% of free radicals



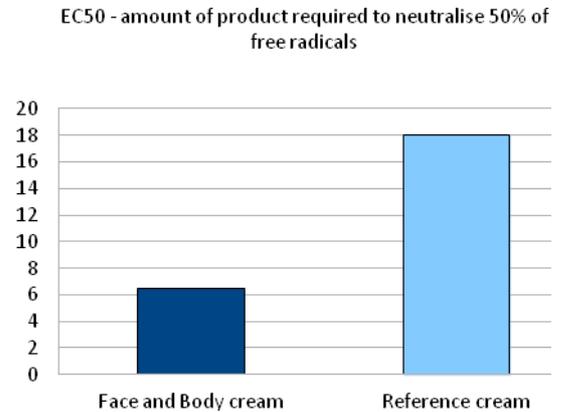
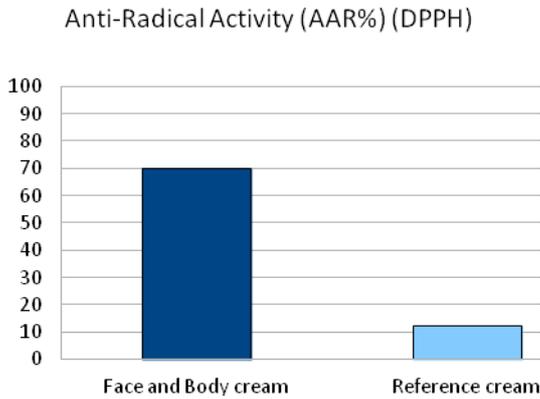
Kinetic of the reaction of the antiradical activity



Test results show that the Mask Scrub has a very high antiradical activity and quantities much lower than the reference cream are sufficient to neutralize free radicals present in the skin. The graph of the kinetic of the reaction also shows how the Mask Scrub tears down the concentration of free radicals effectively and very quickly. The product used as an anti-aging mask, releases anti-free radicals to the skin whilst performing the scrub.

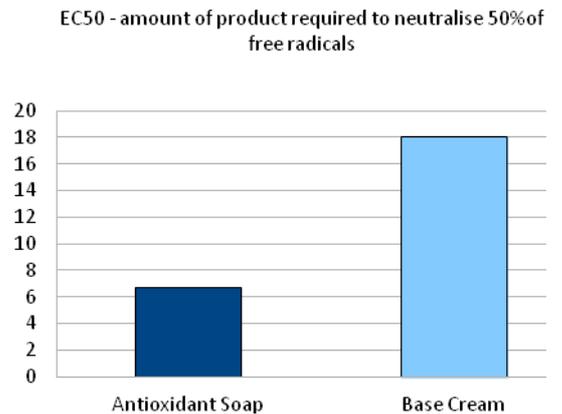
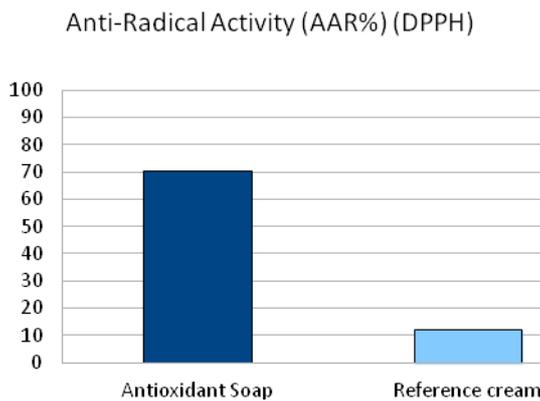


Anti-Age Face and Body Cream Multi-effect Action



Analyses evidence a high anti-radical activity and lower quantities with respect the reference cream sufficient to neutralize free radicals present in the skin. This antiradical activity, combined with the super moisturizing, elasticizing and soothing properties, makes an ideal product for body skin care.

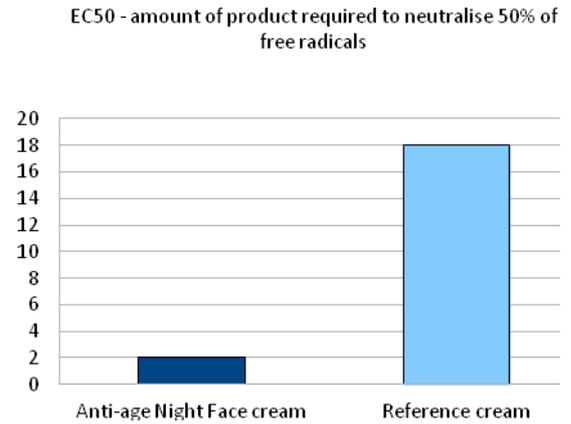
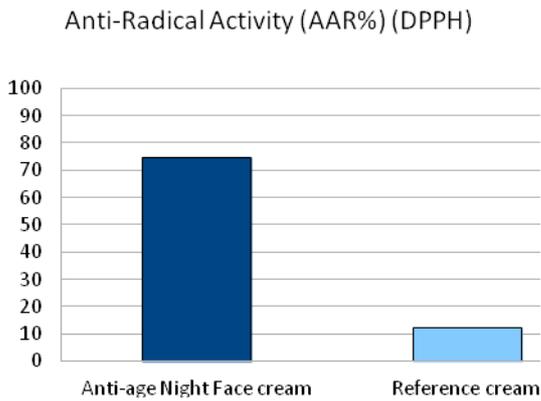
Antioxidant Soap for Face-Body-Hair



Analysis shows a high anti-radical activity and effectiveness from small quantities of product. Since cleaning products permeate the skin easily and quickly, just a short application time (1-3 minutes) produces an anti-radical skin enrichment, along with sebum-regulating and rebalancing activity, which are indicated before an emulsifier treatment.

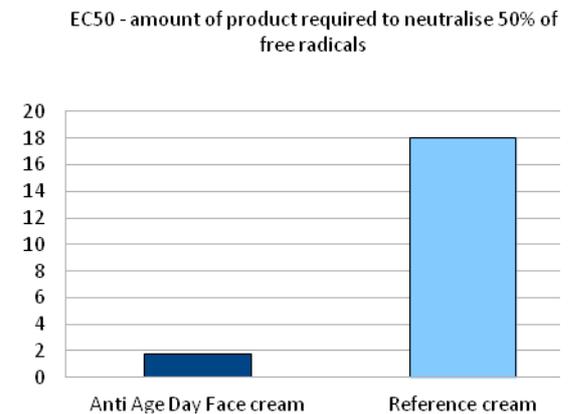
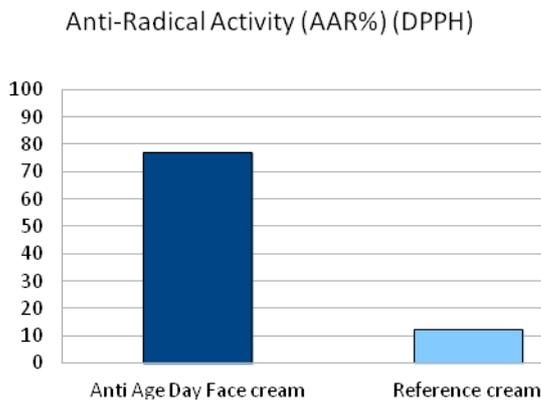


Anti-Age Night Face cream



Analysis shows a high anti-radical activity and amounts much smaller than the base cream required for facing free radicals. This anti-free radical activity, combined with the highly nourishing properties make it the ideal product, slow-releasing, for the regeneration of face skin at night.

Anti-Age Day Face cream



The graph shows that a minimum amount of product is required for the neutralization of free radicals, compared to the reference cream. The high effectiveness in the reduction of free radicals, combined with the moisturizing properties, anti-dehydrating and re-balancing, make it ideal for daytime use and before exposure to UV radiations and other external agents.



These results will be submitted to Journal of Cosmetic Dermatology (Wiley Ed.) as paper research.

Prof. Annalisa Romani

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