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Scientific Research

# Dualistic Properties of Cosmetic Formulations Based on Phenylpropanoids from *Ajuga reptans*

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## ABSTRACT

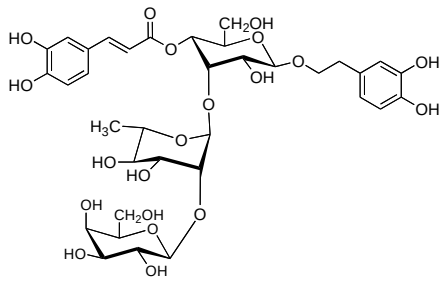
Our continued interest in the research and development of cosmetic active ingredients deriving from natural sources led us to investigate the potential of a purified extract of *Ajuga reptans*, a plant belonging to the family Labiatae and known for its traditional use in skin healing. The extracts deriving from a biotechnology platform are composed by meristematic cell culture, developed in the frame of a NTFP (non-timber forest product) project, and characterized by high content in phenylpropanoid, of which teupolioside represents the majority component. The latter is a phenylpropanoid glucoside, structurally correlated echinacoside and known in the literature for the antioxidant properties. This study was conducted with the purpose of evaluating the applicability of the *Ajuga reptans* extract within different cosmetic formulations. In particular, Photochemiluminescence (PCL) was used to proof the antioxidant capacity of cosmetic formulations containing the product, in relation to the change of the title of teupolioside. Furthermore, UVA and UVB filtering properties were also investigated. The results of the study showed relevantly antioxidant capacity of the finished formulation against superoxide anion, which is the main reactive oxygen species responsible for skin aging and significant synergic capacities to filter UV radiation.

**Keywords:** Phenylpropanoids; Superoxide Anion; Radical Scavenger; UV Filter; Cosmetic Formulation

## 1. Introduction

*Ajuga reptans*, typical of the grassy zones of Europe, Western Asia and Africa, is an annual herbaceous plant belonging to the Lamiaceae family (**Figure 1**).

It is widely present in temperate regions, especially in mountainous areas, where it is frequently used as forage for cattle. *Ajuga reptans* L. is frequently used in the traditional medicine of many countries especially, the eastern part of Europe, for its skin healing properties. The extracts obtained from "bugle" (*Ajuga reptans* L.) most likely own their activity to the content of polyphenols of the flavonoid and polyphenol-carboxylic acids type (antioxidant, vascular and antimicrobial properties), as well as of iridoids (anti-inflammatory and wound healing) [1]. Moreover, the research on cosmetic product for sun protection is evolving towards the development of natural products. In addition to classic UV filters, active ingredients with antioxidant activity can block the activity of reactive oxygen species (ROS) when formed during exposure, and these characteristics would be very interesting if expressed within the same molecule (dualistic properties) [2]. The development of an integrated sun protection system, which would allow taking full advantage of the beneficial influences of sunlight, but reducing skin damages, is becoming more important and essential. The field of solar products (dermoprotective and after-sun) consists in the use of natural emollient, *i.e.* lipid vegetable oils such as coconut, avocado, wheat germ, shea but-




Teupolioside

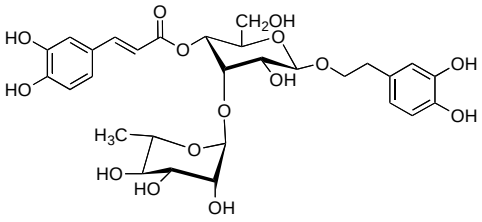
**Figure 1. Structure of teupolioside.**

ter, or of different plant extracts, used for antioxidant activities specific of the plant complex. A few of these ingredients are also endowed by both antioxidant and UV protecting (dualistic) activities [3]. Our continued interest in the research and development of cosmetic ingredients and nutritional supplements, led us to investigate the potential for use in cosmetics of an extract of *Ajuga reptans* obtained with a particular biotechnology platform, employing *in vitro* plant cells cultures. Plant cell culture consents production of the active compounds present in plants, allows the attainment of concentrated extracts in phytoconstituents of the plant, which is usually difficult to obtain with traditional methods (*i.e.* extraction from plants) and is highly sustainable. Extracts obtained from the *Ajuga reptans* cell culture, are characterized by the presence of phenylpropanoids (PPs) and responsible of the activity profile of the plant [4]. As mentioned above, these compounds perform an important protective function facing environmental stress, and in virtue of this, they bear important biological properties. In this study, because of our interest in dualistic molecules, we investtigated the free radical scavenging and UV filtering potential of formulations based on an extract from plant cell lines of *Ajuga reptans*, with high titre (minimum 50%) in total PPs, of which the majority is the type A (PPA) also called teupolioside.

The latter is produced as a secondary metabolite by *Ajuga reptans*, but in our cell cultures, it appears to be the primary component. Smaller amounts of the phenylpropanoid B (PPB, methoxy-teupolioside) and the phenylpropanoid C (PPC, verbascoside) are also present (**Figures 2** and **3**).

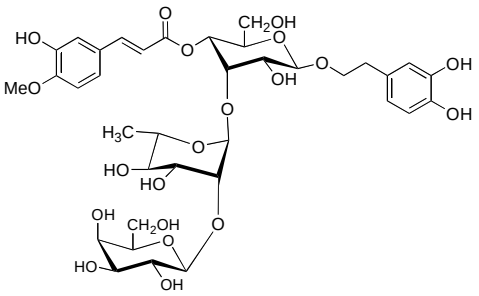
From the structural point of view, the phenylpropanoid glycosides (PPGs) are characterized by a cinnamic acid and a phenylethanolmoyeties bound to a same molecule of glucopyranose (generally glucose), with an ester bond and glycosidic bond respectively [5]. The agliconic portion is responsible for the antioxidant activity [6], antimicrobial, anti-inflammatory and anti-mutagenic whereas the glycosidic part is responsible for hydrosolubility. Moreover, cinnamic acid derivative, due to its structure, possesses interesting protective properties against erythema induced by UVB [7], and on the other hand, phenylethanol is a potent antioxidant. Synthetic derivatives of cinnamic acid are widely used as filters in commercial sun formulations. The reference compound for this category is the octylmethoxycinnamate (Parsol MCX), which represents one of the filters most used in cosmetics, by virtue of moderate cost and low toxicity.

Taking this into account, we focused on the investigation of phenylpropoanoids obtained from *Ajuga reptans*, because of many existing studies on two other related phenylpropanoid glycosides: echinacoside (from *Echinacea angustifolia*), and verbascoside (from *Verbascum sinuatum*). From the "cosmeceutical" point of view, these



Verbascoside

**Figure 2. Structure of verbascoside.**



Methoxy-Teupolioside

**Figure 3. Structure of methoxy-teupolioside.**

molecules are known for the protection of collagen type III, from the degradation caused by oxygen free radicals [8]. The echinacoside possesses *in vivo* healing and anti-inflammatory activity by virtue of anti-hyaluronidase properties, involved in the maturation and organization of the fibrous tissue [9]. Based on these premises, we have undertaken the present study, evaluating 1) the antioxidant capacity of the finished cosmetic formulations, following the approach previously developed by us [10-15], and 2) determining the Sun Protection Factor (SPF) following the directive of Cosmetic Europe [16]. Unexpectedly, antioxidant capacity against superoxideanion, and the main reactive oxygen species, responsible for skinaging, increased during accelerated stability studies. Moreover, the extract obtained was endowed by interesting UV filtering capabilities.

## 2. Stability and Antioxidant Capacity of Cosmetic Formulations

The antioxidant potential of a finished product is usually attributed by the concentration of contained antioxidants, using traditional analytical methods. This evaluation does not take into account a number of variables, *i.e.* possible interactions between the functional ingredients, the presence of so-called "minor ingredients" (compared to the active substances) that compones the formulation. Because all the ingredients in a finished cosmetic product determine the antioxidant effectiveness, we recently developed a new protocol for the instrumental assessment of the antioxidant capacity of the cosmetic products, active ingredients and excipients comprised. Using our ap-

proach and in order to assess the contribution of the ingredients, two different emulsions have been developed. These differ in the kind of ingredients, the first characterized by ingredients chosen for their antioxidant potential, the other with ingredients devoided by this ability. Furthermore, the *Ajuga reptans* extract was inserted at two different percentages (0.3% and 0.6%), to evaluate the variation in the activity at the variation of the concentration of active.

## 2.1. Material and Methods

The extract from cell culture of *Ajuga reptans*, as a yellow powder, water soluble, was kindly supplied by the IRB Srl (Institute for Biotechnological Research, Altavilla Vicentina, Vicenza). The study was carried on two cosmetic bases, termed ALAB and ALAB-AOX, both at different concentrations of extract:

- ALAB-1 and ALAB-AOX-1 containing 0.3% of active;
- ALAB-2 and ALAB-AOX-2 containing 0.6% of active.

The bases tested were the following, ingredient and preservative system have been chosen as simple as possible to avoid interference:

ALAB emulsion (O/W): (formulated with emollients, emulsifiers and other ingredients that do not have antioxidant properties).

INCI: Aqua, paraffinum liquidum, Cetyl Alcohol, Glyceryl stearate, PEG-75 Stearate, Ceteth-20, Steareth-20, Octildodecanol, Cetearyl alcohol, Ciclotetrasiloxane, Dimethicone, Phenethyl Alcohol, Caprylyl Glycol, Xanthan Gum, Disodium EDTA. From the cosmetic technology point of view, is an O/W formulation characterized by the presence of a nonionic emulsifier, able to stabilize the system by the formation of liquid crystals structures. The emollients part is composed by a mixture of mineral oils and silicone. As a stabilizer of the aqueous phase was employed Xanthan gum.

ALAB-AOX emulsion (O/W): (formulated with emollients, emulsifiers and other ingredients that have antioxidant properties)

INCI: Aqua, Olive Oil, Cetearyl Olivate, Sorbitan Olivate, Triticum Vulgare, Limnanthes Alba, Rice Wax, Fitic Acid, Phenethyl Alcohol, Caprylyl Glycol, Xanthan Gum, BHT.

From the cosmetic technology point of view is an emulsion O/W, formulated with a nonionic emulsifier of vegetable origin (contains the oleic fraction of olive oil esterified with cetearyl alcohol and sorbitan). It differs from the formulation ALAB for the presence of components such as rice wax, wheat germ oil, olive oil and Phytic acid was added to the chelating and antioxidants properties.

## 2.2. Analysis

### 2.2.1. HPLC

The different cosmetic formulations were subjected to accelerated aging at 40˚C, and monitored by HPLC in order to assess phenylpropanoids concentration over the time. Quantitative determination of the total phenylpropanoids in *Ajuga reptans* extracts was performed with a Agilent 1100 Series HPLC System equipped with a G1315A DAD and with an Hydro RP18 Sinergi 80A column (4.6 × 150 mm, 4 μm) from Phenomenex. The method involves a run of 20 minutes in isocratic conditions with 0.01 M $H_3PO_4$ in $H_2O$:$CH_3CN$ = 82:18 at room temperature, with a flow 0.8 mL/min. The teupolioside PPA was identified and quantified by the external standard method. All solvents, analyticals and reagents were from Sigma-Aldrich srl, Milan, Italy.

### 2.2.2. Preparation of the Samples

The samples of each emulsion were accurately weighed (500 mg) and diluted in 10 ml mixture of $H_2O$:THF = 50:50. Each sample is shaken in a vortex mixer for about 60 seconds until complete dissolution of the cosmetics matrix. The solutions are then diluted 1:2 with $H_2O$ and each sample is filtered with 0.45 μ cellulose acetate filters. The volume injected into the column for each run was of 20 μl and three injections for each sample were carried out.

### 2.2.3. Photochemiluminescence (PCL)

PCL assay, based on the methodology of Popov and Lewin [14,15], was used to measure the antioxidant activity of extracts with a Photochem® apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photo-sensitizer, when exposed to UV light (Double Bore® phosphor lamp, output 351 nm, 3 m Watt/$cm^2$). The antioxidant activity was measured using both ACW (Antioxidant Capacity of Water soluble substance) (data not shown) and ACL (Antioxidant Capacity of Liposoluble substance) kits provided by the manufacturer designed to measure the antioxidant activity of hydrophilic and lipophilic compounds, respectively [13,14]. For ACW studies, the luminol reagent and Trolox® work solution were freshly prepared according to the ACW protocol. The presence of Trolox® (or any other antioxidants from the extracts) retarded luminescence for a period: hence, a lag time was noted before a signal was measured. The duration of the lag, which is calculated by the computer software from the first derivative of the detector signal at its turning point and intersection with the X-axis, was plotted against the concentration of Trolox® added to the assay medium. The concentration of the added extract solution was such that the generated luminescence fell within the limits of the standard curve. Therefore, the lag time

(seconds) for the ACW assay was used as the radical scavenging activity and the antioxidant capacity calculated by comparison with a Trolox® standard curve and then expressed as micromoles of Trolox® per gram of matter. In ACL studies, the kinetic light emission curve, which exhibits no lag phase, was monitored for 180 s and expressed as micromoles of Trolox® per gram of dry matter. The areas under the curves were calculated using the PCL soft control and analysis software. As greater concentrations of Trolox® working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox®. The observed inhibition of the signal was plotted against the concentration of Trolox® added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The extracts for ACW and ACL measurements were centrifuged (5 min at 16000 g) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 µL of the diluted extract (1:40, v/v) in HPLC-grade water (ACW) or HPLC-grade methanol (ACL) was sufficient to correspond to the standard curve.

#### 2.2.4. Preparation of Samples

An accurately weighed quantity of each formulation previously prepared was suspended in 10 ml of mixture MeOH/esano/$Et_2O$ (1:1:1) sonicated for 20 min at 20˚C and subsequently centrifuged for 5 - 7 min at 3000 rpm. The antioxidant capacity was evaluated by the means of a calibration curve obtained by using as a standard the Trolox® and the results obtained were expressed in Trolox® nmol/mg of cream.

#### 2.2.5. Accelerated Stability Study

The cosmetic formulations were subjected to accelerated aging at 40˚C, and the samples for the analysis were drawn at the following times:

- T0 = at the moment of preparation;
- T1 = one week after preparation (7 days);
- T2 = two weeks after preparation (14 days);
- T3 = one month after preparation (30 days);
- T4 = three months after preparation (90 days).

## 3. UV Filtering Capacity of Cosmetic Formulations

As stated above, the conjugated aromatic portion of teupolioside, presents a structural similarity with that of Parsol MCX. It is thus reasonable that teupolioside can also possess filtering properties against UVB rays. To support this hypothesis we have carried out some preliminary analysis to verify its photoprotective activity and its potential use in solar formulations. Two kind of study have been conducted, 1) stability of the teupolioside against solar radiation by using a solar simulator and 2) determination the SPF factor. At first photostability was assessed, the extract was introduced win different cosmetic formulations, and subjected to a series of radiation intensity and energy equal to that of the sun, to verify the possible alterations. After irradiation the formulations were analyzed in relation to the content of the active principle (teupolioside) and the variation of the antioxidant capacity. The two parameters were monitored before and after irradiation, through HPLC and PCL respectively. After checking the stability of the extract, in order to quantify the possible photoprotective capacity, the value of SPF was determined spectrophotometrically according to the international method of Diffey and Robson [17].

### 3.1. Material and Methods

#### 3.1.1. Simulation of Solar Radiation

The test was performed on cosmetic formulations containing 0.1% of extract with titre >50%, irradiation was continued for two hours in a solar simulator Suntest CPS + (Atlas, Germany), consisting of a Xenon lamp (intensity of the radiant energy emitted: 250 W/$m^2$), with optical filters with IR reflective coating and a UV filter/Suprax, to simulate the conditions of exposure to the sun ($\lambda$ from 300 to 900 nm). Formulations: we designed standard formulations, in order to be able to assess if the vehicle or the different components can in some way influence the degradation of teupolioside. The preservative system has been selected in view of its performance and safety of use. Each product contained 0.1% of extract. The formulations tested are the following:

ALAB1: Hydroxyethylcellulose gel.

INCI: Aqua, Glycerin, Hydroxyethylcellulose, Propylen glycol, Phenethyl Alcohol, Caprylyl Glycol, *Ajugareptans* cell culture extract.

ALAB2: O/W emulsion designed and formulated to avoid possible interactions between the cosmetics matrix and the extract.

The emulsifier used in the formulation gives a good stability and allows easy dispersion of the active principles. The lipophilic component is composed of a mixture of mineral oils and silicone.

INCI: Aqua, Glyceryl Stearate, Ceteareth-20, Ceteareth-12, Cetearyl Alcohol, CetylPalmitate, Dicaprylylcabonate, CetearylIsononyl, Glycerin, Oleylerucate, Phenethyl Alcohol, Caprylyl Glycol, Dimethicone, Disodium EDTA, *Ajugareptans* cell culture extract.

As a reference, an aqueous solution containing 0.1% of cell extract of *Ajuga reptans* was used.

500 mg, accurately weighed, of each cosmetic formulation was sampled, and then distributed in a homogeneous and uniform layer on the bottom of a beaker, whereas for the aqueous solution were accurately weighed 2.5 g of solution.

The samples are then placed inside the analysis chamber of the simulator and irradiated for 2 hours at a temperature of 37˚C.

#### 3.1.2. HPLC

The quantitative determination of the teupolioside in the samples has been carried out as described above, before and after irradiation.

#### 3.1.3. Sample Preparation

The 0.1% solution is diluted 1:50 in $H_2O$; Accurately weighed 500 mg of gel ALAB1 are diluted with 10 ml of $H_2O$; 500 mg accurately weighed O/W ALAB2 emulsion is diluted with 10 ml mixture of $H_2O$:THF = 50:50 and placed under mixing with the aid of a vortex mixer for about 60 seconds until the complete dissolution of the cosmetics matrix. The solutions are next diluted 1:2 with $H_2O$ and filtered with 0.45 μ regenerated cellulose filter before each injection.

#### 3.1.4. Photochemiluminescence (PCL)

Determination of antioxidant capacity of cosmetic formulations was conducted before and after irradiation as described above.

#### 3.1.5. Determination of the SPF *in Vitro*

The test is carried out according to guide line COLIPA 2009 (now Cosmetic Europe), to the recommendation EC 647/2006 of the 22/09/2006 about the efficacy of sun protecting products and to the evaluation of Boots star rating system. Employ a UV-VIS spectrophotometer Shimadzu (Shimadzu Italy, Milan) mod. UV-2600 provided with 60 mm integration sphere ISR-2600Plus and dedicate software package for the determination of SPF/UVA (Sunny Detection) it features dual-beam optical system, single monochromator with spectral range up to 1400 nm with a Czenry-Turner optycalbanch endowed with proprietary correction of chromatic aberration (low-raylight) and UV-Probe software control. Support for the determination is PMMA WW2-2 μm-Plates (SCHÖN-BERG GmbH, Hamburg). Quantity of sample (cosmetic preparation to be analyzed) at a dose of 2 ± 0.04 mg/cm$^2$ follows Cosmetic Europe Guidelines.

The measurement is repeated several times (at least 6) and at the end of the program through acquisitions and analytical processing spectral data by the Sunny Detection package a mean absorbance spectrum was determined.

For the determination of SPF factor the equation Diffey and Robson is applied.

$$\mathrm{SPF} = \sum_{290}^{400} E(\lambda) B(\lambda) \frac{\sum_{290}^{400} E(\lambda) B(\lambda)}{\sum_{290}^{400} (\lambda) E(\lambda) T(\lambda)}$$

where:

$E(\lambda)$ is the spectral irradiance of sunlight, measured at noon in the summer in southern Europe, at latitude 40˚ North, 20˚ zenith angle, thickness of the ozone layer 0.305 cm.

$B(\lambda)$ is the erythema action spectrum of solar radiation, obtained by McKinlay and Diffey comparing over 12 erythema inducing spectra measured between 1929 and 1985.

$T(\lambda)$ is the monochromatic spectral transmittance.

Formulations:

ALAB8: was an hydroxyethyl cellulose gel, functionalized with 4% of the extract of *Ajuga reptans*. This formulation is "neutral" in the respect of SPF because the gel matrix does not have photoprotective activity. The preservative system (Akema, Italy) has been selected in view of its performance and safety of use. [18].

INCI: Aqua, *Ajuga reptans*, Hydroxyethylcellulose, Propylene Glycol, Phenethyl Alcohol, Caprylyl Glycol.

ALAB9 emulsion (O/W): in this formulation have been inserted photoprotective ingredients of natural origin, to assess the value of SPF obtainable using only plant products. Contains ferulic acid complexed in cyclodextrins, Aloe extract, rice bran oil, γ-oryzanol.

INCI: Aqua, Cetyl Alcohol, Glyceryl stearate, PEG-75 Stearate and Ceteth-20, Steareth-20, Ferulic Acid, Cyclodextrin, Cetearyl alcohol, ButyrospermumParkii, Elaeisguineensis, Rice Bran Oil, Tocopheryl Acetate, Buxus Chinensis, Olive Oil, Rice Wax, Aloe Barbadensis, γ-orizanol, Phenethyl Alcohol, Caprylyl Glycol, Xanthan Gum, Fitic Acid.

ALAB10 emulsion (O/W) the same formula described above that is functionalized with 4% of extract of Ajuga (Titred > 50% in PP) to assess the contribution to the final value of the SPF.

INCI: Aqua, Cetyl Alcohol, Glyceryl stearate, PEG-75 Stearate, Ceteth-20, Steareth-20, *Ajuga reptans*, Ferulic Acid, Cyclodextrin, Cetearyl alcohol, Butyrospermum-Parkii, Elaeisguineensis, Rice Bran Oil, Tocopheryl Acetate, BuxusChinensis, Olive oil, Rice Wax, Aloe Barbadensis, Phenethyl Alcohol, Caprylyl Glycol, Xanthan Gum, Fitic Acid.

## 4. Results and Discussion

### 4.1. Antioxidant Activity

Among ACW and ACL values, only this last one was selected, after preliminary evaluations, for the study. As a general consideration we can say that all formulations containing the extract of *Ajuga reptans*, compared with

the respective base, have a very significant antioxidant capacity. As predictable the emulsions containing the highest concentration of extract (0.6%) have the higher antioxidant capacity (**Figure 4**). At a closer analysis is possible to observe that the antioxidant capacity of the formulations under examination, varies in function of the choice of excipients. In fact, the value of the antioxidant capacity (ACL) of the formulation ALAB-1 at T0 (12.6 nmol of Trolox/mg cream), is approximately 32% lower compared to the value of ALABAOX-1 (18.5 nmoles Trolox®). This trend is also found in the formulations at higher concentration (ALAB and ALAB-2-AOX-2).

The values of higher ACL for formulations ALAB-AOX are attributable to the presence of ingredients endowed with significant antioxidants capacity such as phytic acid, rice wax and oils of vegetable origin. However, as can be seen from the **Figure 4**, increasing concentration of extract of *Ajuga reptans*, does not correspond to a proportional increase in antioxidant capacity. In fact, as regards the formulations ALAB, the ALAB-2 despite containing twice extract with respect to ALAB-1 has an ACL value only 13% higher as compared to ALAB-1. It is possible to observe a similar trend also regarding formulations ALAB-AOX. The variation of the antioxidant capacity of the formulations under accelerated ageing and compared with the respective bases, are shown in **Figure 5**. As might be expected the antioxidant capacity at first decreases in function of time.

However, unattendently, the antioxidant capacity of the formulations ALAB-AOX undergoes, in the first week, a reduction greater than that of ALAB but, increases after two weeks. In fact, for example, from T0 to T1, the value of ACL for ALAB-1 goes from 12.6 nmol of Trolox®/mg of cream to 10.62 (which corresponds to a reduction of 10%), whereas for the ALAB-AOX-1 shows a decline of 43%. During the next time interval (T1 to T2), there is a slight increase of the antioxidant activity of ALAB, whereas the ACL value of formulations ALAB-AOX (different from the previous ones for the presence of components with potential antioxidant activity) decreases in both emulsions although to a greater extent in ALAB-AOX-2.

The trend of the variation of the antioxidant capacity continues to be non-linear even in later times. At the final time (T4), however, despite the three months of accelerated aging, all the tested formulations maintain a significant antioxidant capacity. In particular, for the formulations ALAB-AOX, there was a reduction of the values of ACL equal to 65% compared to T0, while the antioxidant capacity of ALAB decreases to a lesser extent (50% compared to the initial value). To complete the study, in parallel HPLC analysis was conducted, to assess the decrease over the time of teupolioside present in the extract. In **Figure 6**, it is shown that the concentration of PPA

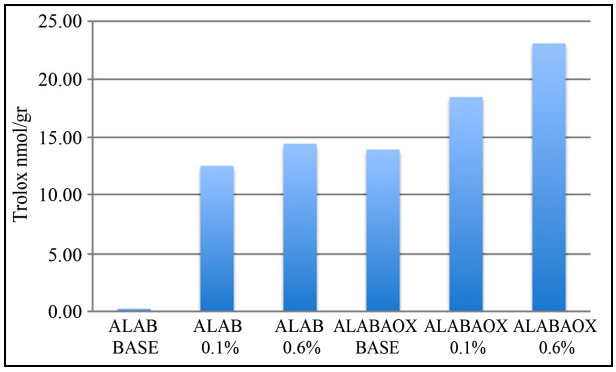



**Figure 4. Antioxidant capacity of formulations based on phenylpropanoids obtained from *Ajuga reptans* (titre > 50% in total phenylpropanoids). The basic formulation was used as a positive control.**

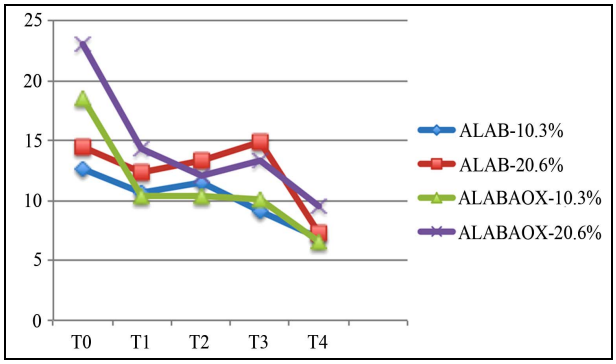



**Figure 5. Variation of the antioxidant capacity cosmetic formulations based extract of *Ajuga reptans* (titre > 50% in total phenylpropanoids) during accelerated aging (40˚C). Legend: T1 = 7 days; T2 = 14 days; T3 = 30 days; T4 = 120 days.**

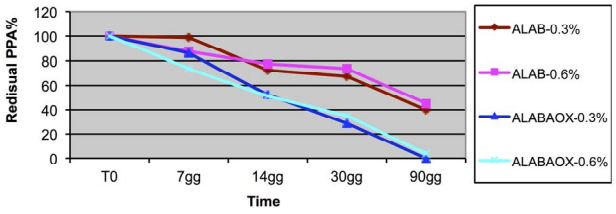



**Figure 6. Change of the concentration of teupolioside in emulsions during accelerated aging (40˚C).**

decreases in function of time. The percentage values of residual PPA, at various times, show, however, that the disappearance of teupolioside is slower and the % recovery greater in ALAB than in the formulations ALAB-AOX. Indeed, at the time of the final observation, in ALAB and ALAB-1 and -2, the amount of active ingredient present is 40% and 45%, respectively, while in ALAB-AOX-2 recovery % of teupolioside is practically zero.

The increase of antioxidant activity at T3 paralleled by disappearance of teupolioside can be explained by the formation of the by-product iso-teupolioside, due to the

migration of caffeic acid moiety from position 4 to position 6 that may occur and is largely dependent from the kind of formulation used [19].

## 4.2. UV Filtering Activity and Stability

### Stability Study

Analyzing the concentration of teupolioside before and after irradiation (**Figure 7**), it is possible to observe that the molecule slowly disappear proportionally in all formulations tested.

In fact, a similar decrease in aqueous solution (6%), in hydroxyethyl cellulose gel (7%) and in the emulsion O/W emulsion (4%) was recorded. Also, the variation of the antioxidant capacity (**Figure 8**) is in line with the amount of decrease of the teupolioside. This finding differs from the trend above observed where the decrease in teupolioside was not accompanied by a concomitant decrease in antioxidant capacity. This behaviour can be explained by the different kind of formulation used. From HPLC, no by-products appeared after irradiation.

The final determination with the solar simulator provides an additional parameter to evaluate the stability of phenylpropanoids upon solar irradiation, which may be different from the behavior observed in solutions.

## 4.3. Determination of *in Vitro* SPF

The Gel ALAB8 (Ajuga 4%) showed a 1.8 SPF, whereas the emulsion ALAB9 6.5 and ALAB10 (Ajuga 4%) 7.2. The values are significant for a natural ingredient based formulation.

The ALAB8 (gel) provides a picture of the ability to absorb UV radiation by the phenylpropanoids. In fact, the lattice of the gel does not influence the absorption of UV, so the radiation is shielded solely by the chemical structure of the phenylpropanoids. The formulation ALAB9, due to the content of natural ingredients with filtering properties, has an interesting value of SPF (6.5). With the addition of 4% extract from cell cultures of *Ajuga reptans*, the value increases to 7.2.

Comparing the value of the gel, with that of the emulsion functionalized with the extract of *Ajuga reptans*, it is clear that the vehicle is critical for the SPF value given by the ingredient. The spectrophotometric determination also allows to check other interesting parameters such as the UVA/UVB ratio (UVA Ratio), between the total absorption in the UVA region (400 - 320 nm) and UVB (320 - 280 nm). This is important to understand whether the formulation provides a good overall protection over the whole range of the UV rays that are involved in sun exposure. Based on the value of the ratio five categories have been defined (**Table 1**) [17].

As for this classification the following classification can be drawn for our formulations (**Table 2**). The extract

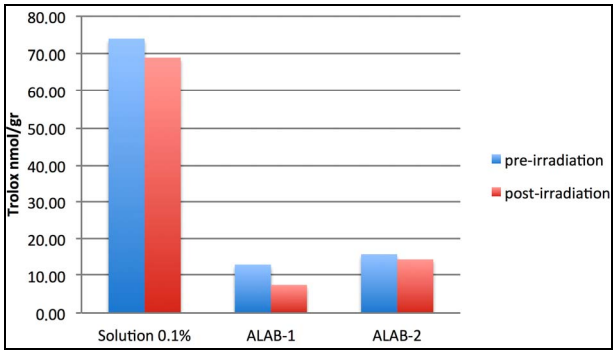



**Figure 7. Concentration of teupolioside before and after irradiation.**

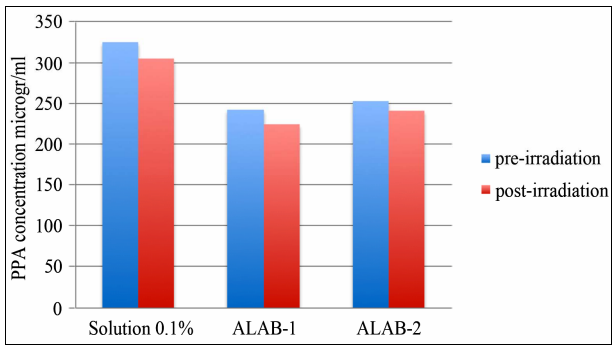



**Figure 8. Antioxidant capacity of the formulations before and after irradiation.**

**Table 1. Classification categories UVA-UVB ratio.**

| UVA Ratio | Star Category | Description |
|---|---|---|
| from 0.0 to <0.2 | - | LOW |
| from 0.2 to <0.4 | * | MODERATE |
| from 0.4 to <0.6 | ** | GOOD |
| from 0.6 to <0.8 | *** | SUPERIOR |
| ≥0.8 | **** | MAXIMUM |

**Table 2. UVA-UVB of formulations analyzed.**

| **Formulation** | Gel ALAB8 Ajuga 4% | Emulsion ALAB9 | Emulsion ALAB10 Ajuga 4% |
|---|---|---|---|
| **UVA Ratio** | 1.2 | 0.77 | 0.98 |
| **Category** | **** | *** | **** |
| **Description** | MAXIMUM | SUPERIOR | MAXIMUM |
| **Cosmetic Europe Standard** | UVA | | UVA |

from cell cultures of *Ajuga reptans* offers not only a coverage over the whole range of UV, as evident from the value obtained for the gel, but can also improve the UVA-UVB ratio. The growing importance of having

preparations that can offer a protection also against UVA prompted us to evaluate a protection factor (data in **Tables 1-3**) that was calculated concomitantly with SPF.

The values are specific for these particular formulations and need to be determined case by case as variations occur in the vehicle, excipients, and emulsifiers. Future studies will be addressed to verify how the vehicle can affect the photostability and the SPF value, and eventual synergies with common filters used in cosmetics consenting lowering of the concentrations of these latter.

## 5. Conclusions

The dermo-cosmetic industry is strongly oriented to the concept of nature as a source of raw materials, with particular attention paid in recent time to renewable and sustainable sources. In this work, we described a study related to a particular type of extract, which is obtained from *in vitro* cultures of plant cells of *Ajuga reptans* and characterized by a high titre in teupolioside, a phenylpropanoid with high antioxidant activity. The innovative nature of this technology platform has attracted our research interest, since the *in vitro* culture enables rapid propagation of selected plants based on the characteristics of the active ingredients of interest present in them. This innovative approach can account for the pharmaceutical, cosmetic and herbal fields; it is a strategic alternative for the supply of active ingredients, which is highly standardized and not directly obtainable by extraction from traditional plant due to the low content (*i.e.* secondary metabolites). A number of studies have been performed in order to evaluate the potential use of this extract in the cosmetic field. The antioxidant capacity of the extract was evaluated, using the technique PCL within prototype formulations at 0.3% - 0.6%. Parallel evaluation of the concentration of phenylpropanoid by HPLC was also conducted leading to the finding that the antioxidant activity is maintained or even increased due to the partial conversion of teupolioside to isoteupolioside over the time.

Due to the presence of a cynnamic acid moiety, UV filtering properties were also investigated disclosing interesting UVB and UVA protecting activity, good UVB/UVA ratio and good photostability. These findings accompained by the potent antioxidat activity which is displayed by finished products indicate a promising role of these extracts in solar product claiming natural composition.

In relation to the possible application in dermatological field and close relationships between echinacoside and teupolioside, the extract from cell cultures of *Ajuga reptans* deserves further investigations as possible anti-inflammatory agent.

In conclusion, the preliminary assessment described here has highlighted new applications of *Ajuga reptans* extracts for the first time. In particular, the quite interesting features confirm that the plant cell cultures are a good method to obtain extracts with high content valuable active molecules, which are found only in trace amounts as plant secondary metabolites. The same technology can also be applied to plants for ethical reasons (rare plants) or difficulty in their extensive growth which can not be collected in large quantities. Such technology finally allows obtaining extracts with a high degree of standardization which are hardly obtained by direct extraction from plants due to the variability of the life cycles and different soils compositions.

**Table 3. Factors of protection against the UVA of the formulations analyzed.**

| Formulation | UVA Protection Factor |
|---|---|
| Gel RB8 Ajuga 4% | 1.5 |
| Emulsion ALAB9 | 3.7 |
| Emulsion ALAB10 Ajuga 4% | 4.2 |

This first phase of work has helped highlight just some of the potential applications of the extract of *Ajuga reptans*, and further studies are currently ongoing with the aim to investigate the possible lenitive and healing properties.

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