Standardized phytocomplex of *Perilla frutescens* derived from *in vitro* cell cultures: maintenance of skin integrity and use in vaginal gel formulations

Pressi, Giovanna[1*]; Rigillo, Giovanna[2]; Governa, Paolo[3]; Biagi, Marco[4]; Borgonetti Vittoria[5]; Baini Giulia[4]; Guarnerio, Chiara[1]; Bertaiola, Oriana[1]; Frigo, Marco[1]; Merlin, Matilde[1]; Paltrinieri, Stefania[1]; Barbieri, Elisa[1]; Semenzato, Alessandra[5]

[1] Aethera Biotech s.r.l., Camisano Vicentino (VI), Italy; [2] Department of Biomedical, Metabolic and Neural Science, University of Modena and Reggio Emilia, Italy; [3] Department of Biotechnology, Chemistry and Pharmacy – Department of Excellence 2018-2022, University of Siena, Italy; [4] Department of Physical Sciences, University of Siena, Earth and Environment; [5] Department of Neuroscience, Psychology, Drug Research and Child Health (NEUROFARBA), University of Florence, Italy; Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy.

* Giovanna Pressi, via dell'Innovazione 1 Camisano Vicentino (VI) Italy, +39 0444 419451 giovannapressi@aetherabiotech.it

Introduction

Plant cell culture technology is a technique for growing of plant cells under strictly controlled environmental conditions that makes it possible to provide preparations with a standardized content of active substances and with a high safety profile for the consumer. *Perilla frutescens* L.Britton, also known as Shiso, is a species of *Perilla* that belongs to the Lamiaceae family, commonly used as an aromatic and medical plant. Perilla has historical importance in Chinese medicine, as it was recorded as a drug around 500 A.D. Some perilla-based products are widely used to treat seasonal allergy symptoms and aphthae. The presence of phenolic compounds, flavonoids, and anthocyanins in *P. frutescens* is amply documented in the scientific literature [1]. A high variability in the content of anthocyanins and polyphenols in said plants is associated with many factors which are difficult to control: seasons, plant age, geographical growing areas and different parts of the plant used for the preparation of products. The preparation of standardized plant derivatives (i.e. extracts with a reproducible metabolite of metabolites) poses numerous problems tied to the variability of the metabolite content in different plant tissues. One method for obtaining contaminant-free standardized plant phytocomplexes in industrial quantities is to use *in vitro* cell cultures. This technology makes it possible to solve the problems tied to the variability of plant extracts, since it provides preparations with a content of active substances that can be reproduced in a standardized manner. *P. frutescens* phyocomplex (PFP), derived from *in vitro* plant cell cultures, has a high and standardized content of rosmarinic acid and anthocyanins and can be used as a new active ingredient for cosmetic and intimate products thanks to its anti-inflammatory and repairing activity. The active ingredient looks like a powder finely dispersed in glycerin and its compatibility with cosmetic raw materials was investigated through gel and gel-cream formulations and its rheological and stability test. The object of this study is to demonstrate the activity of this phytocomplex to maintain vaginal mucosa integrity and its application in intimate gel formulations.

Materials and Methods

Perilla frutescens cell culture

In this study, plants of *Perilla frutescens* L. Britton were used as starting plant material to generate a cell culture and was bought and certified from the nursery plant "Le Georgiche", Brescia, Italy. The botanical species

authentication of *Perilla officinalis* L. was ensured and confirmed through molecular biology analysis (DNA fingerprint) performed in collaboration with Padano Technology Park, Lodi, Italy [2]. The young leaves of *P. frutescens* were washed under running water and sterilized by means of a treatment in sequence with 70% (v/v) ethanol (Honeywell, Wunstorfer Straße 40, D-30926 Seelze, Germany)) in water for about 1 minute, 2% (v/v) of sodium hypochlorite solution (6-14% active chlorine, (MERCK KGaA, 64271 Darmstadt, Germany) and 0.1% (v/v) Tween 20 (Duchefa, Postbus 809, 2003 RV-Haarlem, The Netherlands) for 2-3 minutes and, finally, at least 3 washes with sterile distilled water. The sanitized plant tissue has been cut into minute fragments (explants) and were deposited in Petri dishes containing solidified nutrient medium Gamborg B5 [3] with different combinations and concentrations of plant growth regulators (2,4 dichlorophenoxyacetic acid with and without 6-benzylaminopurine, 2,4 dichlorophenoxyacetic acid with and without kinetin, naphthalene acetic acid with and without kinetin, indole 3-acetic acid with and without Kinetin, naphthalene acetic acid and indole 3-acetic acid with and without kinetin and Picloram with and without 6-benzylaminopurine) and incubated at 25°C and in dark condition. The highest rate of callus growth was observed using the solid medium Gamborg B5 supplemented with 20 g/L sucrose (Sudzucker), 0.9% (w/v) of Plant Agar (Duchefa), 0.5 mg/L of (NAA) naphthalene acetic acid (Duchefa), 1 mg/L of (IAA) indole 3-acetic acid and at pH 6.5 (PFr medium). Calli grown on PFr medium, were subjected to subculture for at least 6 months until they become friable and homogeneous, with a constant growth rate (*Perilla frutescens* stable cell line).

Cell suspension cultures were generated by transferring 10% (w/v) of selected callus into 250 mL of liquid culture medium Gamborg B5 supplemented with 20 g/L sucrose (Sudzucker), 0.5 mg/L of NAA (Duchefa) and 1 mg/L of IAA (Duchefa). The pH was adjusted to 6.5 before autoclaving (PFr liquid medium). The suspension cultures were maintained in climatic room, in dark condition, at 25°C on rotary shaker in constant agitation at 120 rpm and were subcultured in a new liquid medium every 14 days of fermentation. Afterward, to produce large quantities of biomass, the suspension culture was transferred and adapted to growth in bioreactor of progressively increasing size (3L and 5L volume) with an amount of cell suspension inoculated into the liquid medium equal to 12% V/V. To increase the content of rosmarinic acid (RA) and total anthocyanins, after 14 days of fermentation in PFr liquid medium, the cell suspension was transferred to a final liquid medium (Gamborg B5 with the addition of 50 g/L of sucrose, 0.3 mg/L of NAA and 0.8 mg/L of IAA. The pH was adjusted to 5.9 before autoclaving (PFr final liquid medium). The suspensions culture was grown for a culture cycle of 14 days in climatic room at 25°C on rotary shaker in constant agitation at 120 rpm and in dark condition.

Phytocomplex preparation from *Perilla frutescens* selected cell culture

After 21 days of growth in PFr final liquid medium, at 25°C and in the dark, the *P. frutescens* cell suspensions were filtered by 50 µm mesh filter and the medium cultures were discarded. Cells were washed with a double volume of saline solution (0.9% w/v NaCl in sterile water) and added with 1.5% (w/w) of citric acid and then homogenized with ultraturrax at 15000 rpm for 20 minutes. The biomass of homogenated cells was dried using a Mini Spray Dryer (BUCHI-B290) to obtain a powder of PFP with a high content of rosmarinic acid (RA) and anthocyanins [4].

UPLC-DAD analysis

100 mg of powder of the PFP were weighed into a 15 mL test tube and 30 volumes of ethanol/water (60:40 v/v) were added for the quantification of RA and of total polyphenols expresses as equivalent in RA. Instead for the quantification of the total anthocyanins 100 mg of PFP were weight into a 15 mL test tube and 30 volumes of ethanol/water/12N hydrochloric acid (60:39:1 v/v/v) were added. The procedure described below was used both for the quantification of total polyphenols expressed as RA equivalent, for RA and for that of anthocyanins.

The suspension was mixed for 30 seconds with a vortex mixer and sonicated for 15 minutes in an ice bath; finally, it was centrifuged at 4000 rpm for 15 minutes at 6°C. At the end of centrifugation, the supernatant was recovered. 15 mL of supernatant were transferred into a new test tube 15 and preserved in ice until loading into the UPLC system. The sample was diluted 1:10 (first 1:5 in a solvent and then 1:2 in water). The diluted sample was filtered over 0.22 µm filters before being loaded into the UPLC system. Five independent replicates of PFP were extracted and measured. The chromatography system used for quantification of the RA consists in an Acquity UPLC BEH C18 1.7 µm column, size 2.1x100 mm, coupled to an Acquity UPLC BEH C18 1.7 µm VanGuard Pre-Column 3/Pk, size 2.1x5 mm. The platform used for the UPLC-DAD analysis comprises a UPLC system (Waters) consisting of an eluent management module, Binary Solvent Manager model I Class, and an auto-sampler, Sample Manager – FTN model I Class, coupled to a PDA eλ diode array detector. Empower 3 (Waters) software was used to acquire and analyse the data. The chromatography method used was the following: solvent A: water, 0.1% formic acid; solvent B: 100% acetonitrile. The initial condition is 99% solvent A; moreover, the flow remains constant at 0.350 mL/min throughout the duration of the analysis. The chromatography column was temperature controlled at 30°C. Elution of the molecules was conducted by alternating gradient and isocratic phases, as indicated in Table 1.

Time from start of the analysis (minutes)	Percentage of solvent B	Slope
0	1%	
1	1%	linear
11	40%	linear
12	100%	linear
13	100%	Linear
13.10	1%	linear
15	1%	linear

Table 1. Elution of the molecules in UPLC-DAD analysis

Quantification of total polyphenols and RA in the samples were based on absorbance UV/VIS spectra measured at 330 nm. The amount of RA and the total polyphenols (expressed as equivalent of RA) were

evaluated through the comparison with calibration curves obtained from serial dilution of the authentic commercial standard of RA (CAS 20283-92-5; purity≥95%; Sigma Aldrich). The data analysis was carried out with Empower 3 software.

Quantification of total anthocyanins in the samples were based on absorbance UV/VIS spectra measured at 520 nm. The content of total anthocyanins (expressed as equivalent of cyanidine-3-O-glucoside) were evaluated through the comparison with calibration curves obtained from serial dilution of the authentic commercial standard of cyanidine-3-O-glucoside (CAS 7084-24-4; purity≥96%; Extrasynthese). The data analysis was carried out with Empower 3 software.

Evaluation of biological activity by *in vitro* tests

Anti-inflammatory activity

The anti-inflammatory activity test was performed by treating for 24 hours with PFP human keratinocytes simultaneously stimulated with a medium derived from the culture of human monocytes (THP-1) activated with Gram - bacterial lipopolysaccharide (LPS), as a simulation of a condition acute dermatitis. The inflammatory parameters measured by means of ELISA kits were the cytokines TNF-α, IL-1β, IL-6.

Tight junction evaluation

Immortalized keratinocytes were seeded in 24-well plates and grown for 24h. Cells were pre-treated for 2 hours with PFP (100 µg/mL), then stimulated with LPS (250 µg/ml) and H_2O_2 (2mM) for 3 hours. After, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Following permeabilization with 0.5% PBS-Triton X-100 for 10 min, and incubation with blocking buffer (PBS, containing 1% BSA) for 1 hr at RT, cells were incubated at 4°C overnight with primary antibodies anti-ZO-1 (1:100, Genetex), anti-occludin (1:100, Genetex). Cells were washed and incubated with secondary antibodies labeled with Invitrogen Alexa Fluor 488 (Thermo Fisher Scientific, MA) for 1 hr at RT. Finally, staining with DAPI was performed for 10 min and the slide mounted coverslip. The visualization and acquisition of images was performed under the microscope a EVOS fluorescence (Thermo Fisher Scientific, MA).

Skin barrier protein evaluation

The Western Blot (WB) assay allows the analysis of the expression levels of the proteins of interest. $1x10^5$ cells were seeded in 12-well plates and grown for 24h. Cells were pre-treated for 2 hours with PFP (100 µg/mL), then stimulated with LPS (250 µg/mL) and H_2O_2 (2mM) for 3 hours. Cells were trypsinized, harvested and lysed in Laemmly buffer (25 mM Tris–HCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue) after washing with PBS. Protein extracts were quantified, and equivalent amounts of extracts were resolved by SDS–polyacrylamide gel electrophoresis (PAGE), electrotransferred to nitrocellulose membrane (Amersham, UK). The membrane was first blocked for 2 hours with 5% skim milk in 1x TBS and incubated overnight at 4 °C with primary antibodies anti-filaggrin (1:1000, Genetex), anti-loricrin (1:1000, Genetex) in blocking buffer. The following day, the membrane was incubated with anti-rabbit IgG-HRP-linked (1:5000, Cell Signaling, MA) for all targets after washes with TBS-tween. Bands were detected using Immobilon Western Chemiluminescent HRP (Millipore, MA) and detected by

means Imager instrument (Amersham, UK). Quantification of signal optical density was performed by ImageJ software.

Cosmetic formulations with *Perilla frutescens* phytocomplex

To study the compatibility of PFP it was insert in gel formulation using different classes of rheology modifiers: natural (Rheozan® SH, INCI name: *Succinoglycan gum*; Actigum™ CS11 QD, INCI name: *Sclerotium gum*), semi-synthetic (ESAFLOR® HM 22, INCI name: *C18-C22 Hydroxyalkyl Hydroxypropyl Guar)*, and synthetic (Carbopol® Ultrez10, INCI name*: Carbomer)*.

In table 2 is reported the reference formula of gel formulations:

Phase	Ingredients	Composition (%)
A	Water	Add until reach 100
	Glycerin	3 - 0
	Tamarindus Indica Seed Polysaccharide	0.5
	Rheology Modifier	Varies
	PFP glycerin suspension	0 - 3
B	*Phenoxyethanol, Ethyhexylglycerin*	0.9
C	Buffering Agent	Add until reaching pH 5-5.5

Table 2: Reference formula of gels.

Gel formulations were prepared with different concentrations of each rheology modifier to find the right viscosity to maintain the active ingredient in suspension: Rheozan SH (R) at 0.5 – 0.75 and 1%; Actigum CS11 (A) at 1 – 1.5 – 2 – 2.5%; Esaflor HM22 (E) at 0.5 – 0.75 – 1%; Carbopol Ultrez10 (U) at 0.25 – 0.5 – 0.75%.

Water was weighed and heated at 60°C, glycerin was mixed with the rheology modifiers and added to the water under light stirrer. PFP glycerin suspension was added and mixed with a light stirrer. The preservative (Phase B) was added to phase A. Once the gel was cooled down, the pH was measured and adjusted with phase C, if necessary.

The active ingredient was added also in gel-cream formulations according with the reference formula in table 3:

Phase	Ingredients	Composition (%)
A	Water	Add until reach 100
	Glycerin	3 - 0
	Esaflor HM22	0.3 – 0.4 – 0.5
	Rheology Modifier	0.2
	Citric acid	0.0075
B	*Propylheptyl Caprylate*	2
	Caprylic/capric triglyceride	0.5
C	PFP glycerin suspension	0 - 3

D	*Phenoxyethanol, Ethyhexylglycerin*	0,9

Table 3: Reference formula of Gel-creams.

Water was weighed, glycerin was mixed with the rheology modifiers and added to the water under light stirrer. Phase B was added to phase A under stirrer of 4500 rpm for five minutes. PFP glycerin suspension (phase C) was added and mixed with light stirrer until well dispersed. The preservative (Phase D) was added. Once the gel was cooled down, the pH was measured and adjusted with phase C, if necessary.

Rheological analysis

The rheological analysis was performed by using the RheoPlus Anton Paar Modular Compact Rheometer 302-Evolution (MCR 302-E) regulated by a thermostat at 23°C±0,05°C, using CP50-1 (cone/plate) cone and plate geometry with flat surface, a dimeter of 49.55mm, and a fixed gap of 0.115mm. Each sample was subjected to analysis in oscillatory motion, being non-destructive allow to analyze the structural characteristics of the product. The general conditions set for each sample analyzed in the oscillatory motion are indicated in table 4.

Oscillatory Test	**γ (%)**	**ω (Hz)**
Amplitude sweep (AS)	0.01-1000	1
Frequency Sweep (FS)	$\gamma < \gamma_L$	10-0.01

Table 4: condition of the rheological analysis. γ_L is the limit of the linear viscoelastic region (LVE).

Amplitude sweep (AS) describes the deformation behavior of the samples in the non-destructive deformation range, keeping the frequency at a constant value. It is first carried out to determine the limit of the LVE region, that indicates the range in which the frequency can be modified without destroying the structure of the sample. In frequency sweep (FS) the oscillatory frequency is increased while keeping the shear strain constant. The measurements are carried out at a strain level within the LVE region.

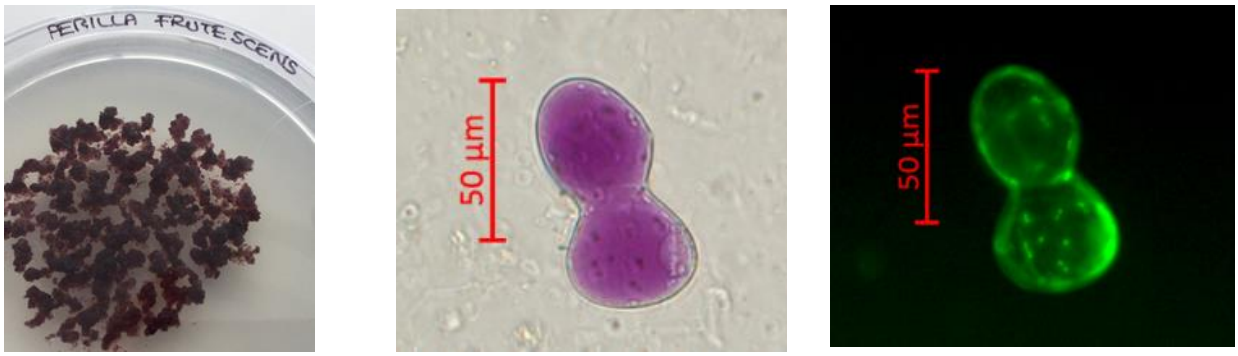
Stability test

The stability test was done by using the centrifuge MPW-56 of MED, at 4800 RPM for 30 minutes.

Results

***Perilla frutescens* phytocomplex was obtained by a stable and selected cell line**

A stable and selected cell line of *P. frutescens* was obtained using the PFr solid medium (medium Gamborg B5 supplemented with 20 g/L sucrose, 0.9% (w/v) of Plant Agar, 0.5 mg/L of NAA, 1 mg/L of IAA at pH 6.5). In this selected solid medium, the *P. frutescens* cell line is pale purple coloured and has a friable texture and with high rate of growth (subculture in fresh solid medium every 21 days). Figure 1 shows the cell culture maintained in solid PFr medium (a) and cells of the line seen under an AXIO-Imager A2 optical microscope (ZEISS), in the bright field mode (b) and after staining with fluorescein diacetate (c).



(a) **(b)** **(c)**

Figure 1. *Perilla frutescens* cell culture maintained in solid PFr medium: (**a**) cells of the *Perilla frutescens* seen under an AXIO-Imager A2 optical microscope (ZEISS), in the bright field mode (**b**) and after staining with fluorescein diacetate (**c**).

The content of RA, total polyphenols and total anthocyanins into cell line were optimized using a PFr final liquid culture medium with a higher content of sucrose (50 g/L) and with a lower concentration of growth hormones (0.3 mg/L of NAA and 0.8 mg/L of IAA). The cells grown into PFr final liquid culture medium were used to prepare the PFP.

The UPLC-DAD analysis was used to estimate the content of RA, total polyphenols, and the content of anthocyanins in the PFP. Total polyphenols expressed as equivalent of RA were quantified by compare of peak areas measured at 330 nm wavelength against the areas of the calibration curve of the reference standard: RA. The RA content was quantified by compare of peak area at retention time 7.5 minutes measured at 330 nm wavelength against the area of the calibration curve of the reference standard. The chromatographic profile of the PFP at the wavelength of 330 nm is shown in Fig 2.

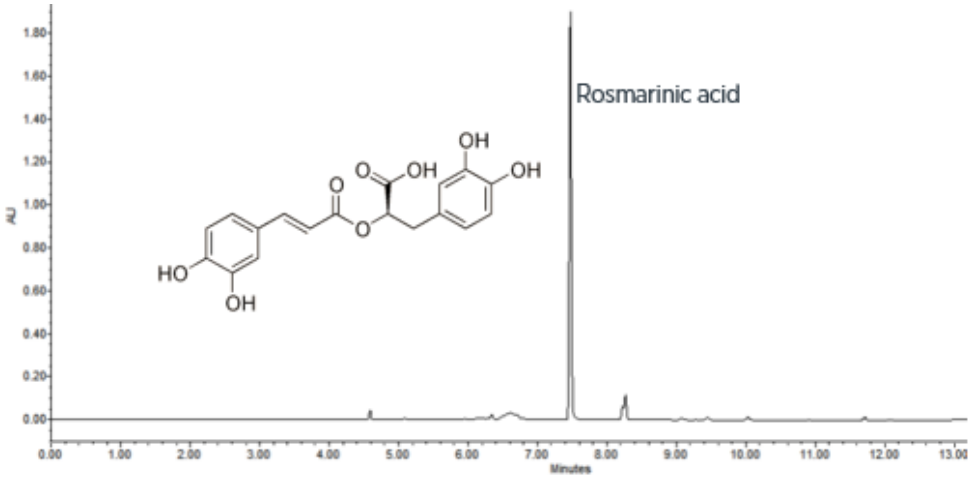


Figure 2. Chromatographic profile of the PFP extract. The main peak at retention time 7.5 min corresponds to RA.

The content of total polyphenols identifies by their characteristic spectrum with λ_{max} at 330 nm and expressed as equivalent of RA was 2.35 ± 0.16% w/w the content of RA, calculated measuring the peak area at retention

time 7.5, was 2.03± 0.16% w/w. Thus, the main component of total polyphenols in the MD is represented by RA. Total anthocyanins expressed as equivalent of cyanidine-3-O-glucoside were quantified by compare of peak areas measured at 520 nm wavelength against the areas of the calibration curve of the reference standard: cyanidine-3-O-glucoside. The content of total anthocyanins identifies by their characteristic spectrum with λ_{max} at 520 nm and expressed as equivalent of cyanidine-3-O-glucoside was 0.10 ± 0.02% w/w.

Evaluation of biological activity by *in vitro* tests

Anti-inflammatory activity

The experimental model developed showed that all inflammatory cytokines dosed undergo upregulation when keratinocytes are stimulated with conditioned medium. The TNF-α undergoes an approximately 10-fold increase in expression compared to the control, while IL-1β and IL-6 increase by about 2 times. The most interesting finding was its surprising effectiveness in inhibiting the release of pro-inflammatory cytokines in keratinocytes stimulated at the highest dosage tested, 100 µg / mL. PFP has been confirmed as a product with a high anti-inflammatory capacity, also in the sample obtained for biotechnology and the over-release of TNF-α, IL-1β and IL-6 was almost completely inhibited by the treatment with the plant sample.

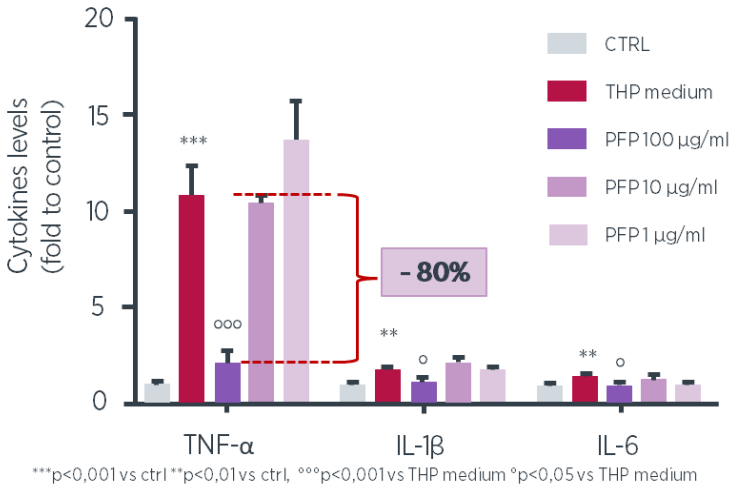


Figure 3. Cytokine dosage on keratinocytes stimulated and treated with PFP.

Tight junction evaluation

Immunofluorescence staining analysis revealed the compromise of ZO-1 junction expression in the keratinocytes following stimulus with LPS + H_2O_2 for 3 hours compared to the control. Treatment with PFP alone did not affect ZO-1 expression, while it was able to prevent damage induced by LPS+H_2O_2 by preserving ZO-1 expression in keratinocytes. Similarly, the expression of the tight junction occludin did not change in unstimulated keratinocytes following treatment with PFP (Figure 4a). The 2-hour pre-treatment with PFP was able to preserve occludin expression impairment induced by LPS+H_2O_2 (Figure 4b).

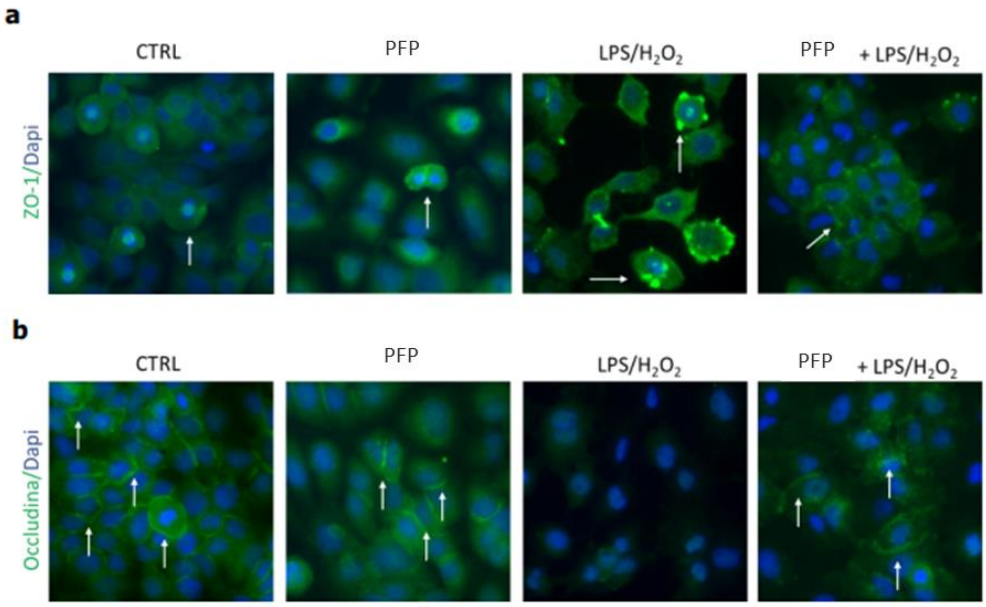


Figure 4b. Immunofluorescence staining analysis revealed the compromise of ZO-1 and occluding junction expression in the keratinocytes following stimulus with LPS + H_2O_2 for 3 hours compared to the control.

Skin barrier protein evaluation

The expression of filaggrin and loricrin, two of the main proteins involved in the formation of the skin barrier at the level of the epidermis, was analyzed. The western blot assay performed on keratinocytes showed a significant increase in the expression of both filaggrin and loricrin after 3 hours of LPS+H_2O_2 stimulation. It is interesting to note how PFP 100 μg/mL stimulated the protein expression of filaggrin and loricrin when administered individually. PFP showed a tendency to reduce the filaggrin and loricrin high expression induced by LPS+H_2O_2, even if the variation is not statistically significant (Fig. 5a, b).

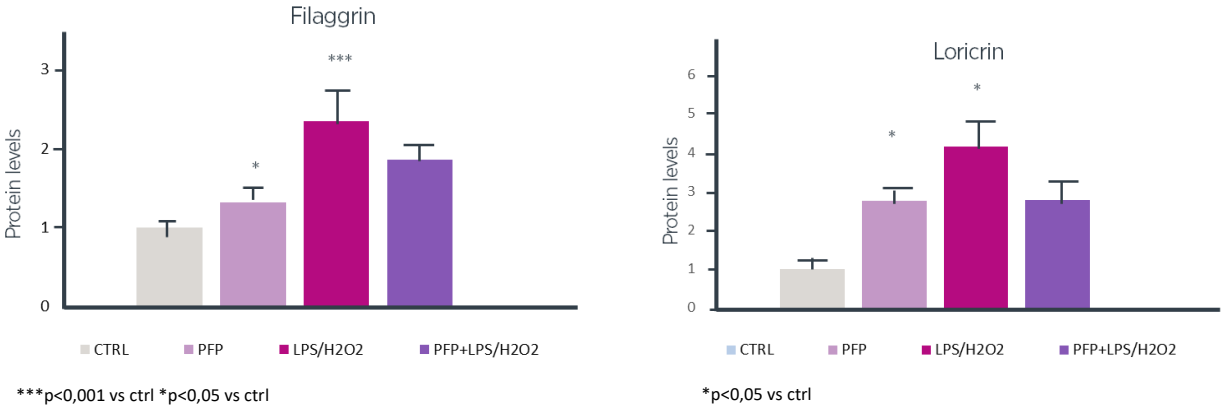


Figure 5. Quantification of the optical density of the levels of filaggrin (a) and loricrin (b), obtained by western blot, in HaCaT cells pretreated for 2 hours with Perilla and stimulated with LPS (250 ng / ml) + H2O2 (2mM). Student T-test statistical analysis (* p <0.05 vs CTRL).

Gel formulations with *Perilla frutescens* phytocomplex

Gel formulations were prepared with different concentrations of each rheology modifier to find the right viscosity to maintain the active ingredient in suspension. Centrifuge test was carried out to evaluate the stability of the gels: the most stable gel formulations were the one with Rheozan SH at 1%, Actigum CS11 at 2.5% and all the gels with Carbopol Ultrez 10, as reported in table 5.

The gels that failed the centrifuge test had a slight precipitate of the powder of the active ingredient PFP.

POLYMER COMMERCIAL NAME	POLYMER SIGN	POLYMER INCI NAME	POLYMER (% w/w)	PFP glycerin suspension (% w/w)	CENTRIFUGE TEST STABILITY
Rheozan SH	**R**	*Succinoglycan gum*	0.5	3	✗
			0.75	3	✗
			1	**3**	✓
Actigum CS11	**A**	*Sclerotium gum*	1	3	✗
			1.5	3	✗
			2	3	✗
			2.5	**3**	✓
Esaflor HM22	**E**	*C18-22 Hydroxyalkyl hydroxypropyl guar*	0.5	3	✗
			0.75	3	✗
			1	3	✗
Carbopol Ultrez 10	**U**	*Acrylates/C10-30 alkyl acrylate crosspolymer (Carbomer)*	**0.25**	**3**	✓
			0.5	3	✓
			0.75	3	✓

Table 5. Summary of the gel formulations and results of centrifuge stability test.

For Carbopol Ultrez 10 was chosen the concentration of 0.25% because other gels had a higher viscosity. Since it is preferred to formulate with natural polymers Carbopol Ultrez 10, despite having stable formulations at all concentrations, it has been eliminated from the preliminary screening. Gel formulations with Esaflor HM22 were not stable. Rheological analyses were done on the selected samples.

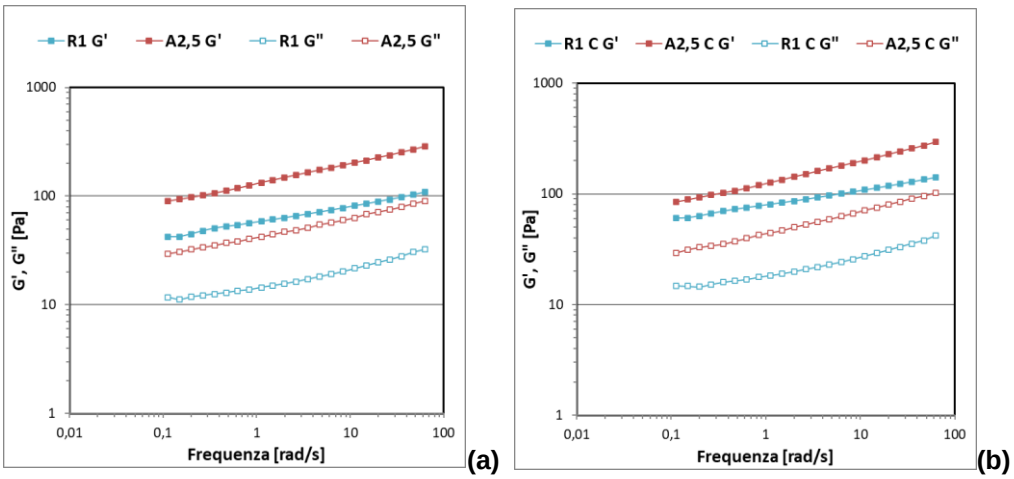


Figure 6. Frequency sweep analysis of gel formulations with Rheozan SH (R) and Actigum CS11 (A) without (b) and with (a) PFP glycerin suspension (C)

FS analysis was performed at a strain within the LVE region, previously determined by Amplitude sweep (AS) analysis, increasing the oscillation frequency and maintaining the amplitude strain constant. The FS analysis demonstrated over the entire frequency range the G' curve above the G" curve and viscosity values between 10 and 1000 Pa, that indicates a typical weak-gel structure. There were no notable differences between the values of the elastic modulus G' nor the viscous modulus G" with and without the active ingredient.

Since Esaflor HM22 (E) is a polymer with emulsifying properties, it was used as an emulsifier alone and in association with other rheological modifiers under study to formulate light gel-creams. Figure 7 shows the frequency sweeps analysis of the samples prepared using Esaflor HM22 (E) alone and with the addition of Tamarind Seed Polysaccharides (T) at 0.2% and the active ingredient (C) at 3%, maintaining the same concentration of total polymers respectively at 0.5% (a), 0.6% (b) and 0.7% (c). It can be notice that by adding Tamarind Seed Polysaccharide (T), a polymer widely used for intimate areas due to its moisturizing and mucoadhesive properties, a decrease of the viscoelastic properties can be detected. The presence of the active leads to an increase in both viscous and elastic modulus compared to the sample without PFP more pronounced in those samples prepared with the lower concentration of polymers.

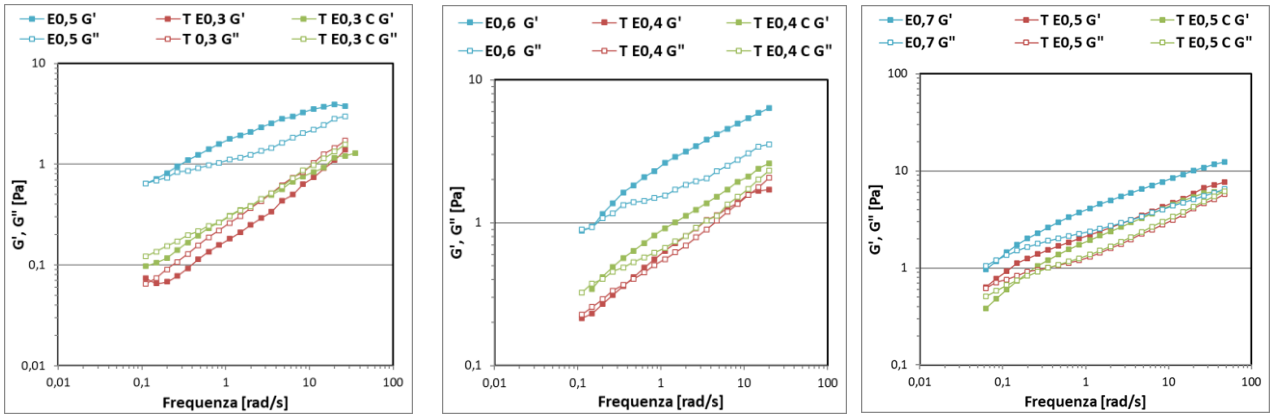


Figure 7. Frequency sweep analysis of samples with Esaflor HM22 (E) alone, comparing with the same samples added with Tamarind Seed Polysaccharides (T) at 0.2% and with the active ingredient (C) at 3%, maintaining the same concentration of total polymers respectively at 0.5% (a), 0.6% (b) and 0.7% (c).

Cream gel formulations with 0.5% of Esaflor HM22 with *Succinoglycan gum (R)* or *Sclerotium gum* (A) at 0.2% at a total polymer concentration of 0.7%, were also prepared and investigated. In Figure 8 the trend of tangent delta in function of frequency of the different gel-cream samples are shown. Tangent delta (tan δ) is a rheological parameter that describes the ratio between the viscous and the elastic modulus; values of tan δ < 1 correspond to viscoelastic or gel-like behaviour. The lower the tan δ, the greater the influence of the elastic component. The addition of both *Succinoglycan gum* (R) or *Scelrotium gum* (A) at 0.2% in the gel formulation prepared with Esaflor HM22 (total polymer amount 0.7%) increases the elastic character of the products respect to the sample prepared using Esaflor HM22 alone (E) or that prepared with Tamarind Seed Polysaccharides (T) (Figure 8a), while a slight increase in the viscosity can be notice by adding the active ingredient in all the samples (Figure 8b). All these samples were stable at the centrifuge test.

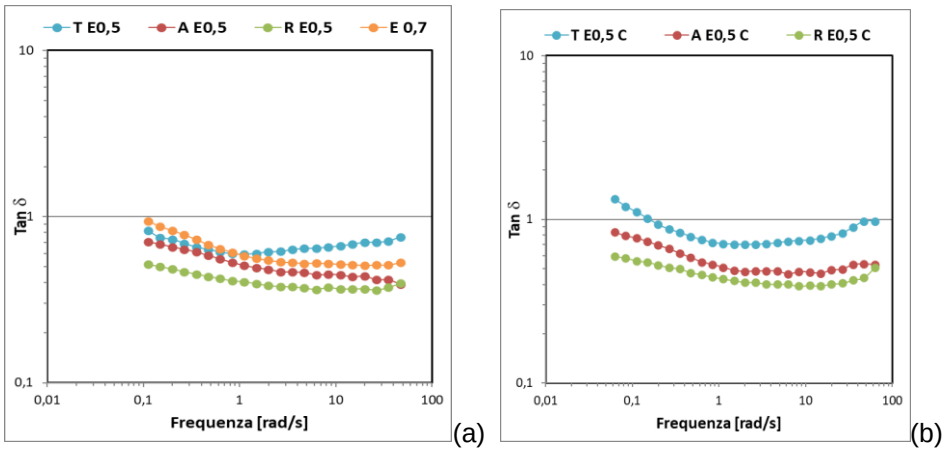


Figure 8. Tan δ values in function of frequency of gel-cream samples with 0.5% of Esaflor HM22 (E) with *Succinoglycan gum* (R) or *Sclerotium gum* (A) at 0.2% at a total polymer concentration of 0.7 without (a) and with (b) PFP glycerin suspension.

Keeping fixed the total amount of polymer concentration at 0.7% we decide to prepare samples by using ternary mixture of polymers in order to obtain the best fit in terms of stability of the product, sensorial properties and mucoadhesive properties. Esaflor HM 22 (E) is the emulsifier polymer that allows to create the product's structure, Tamarind Seed Polysaccharide (T) is the polymer that can guarantee mucoadhesion, while *Succinoglycan gum (R)* or *Scelrotium gum* (A) are two polymers that can optimize the stability of the product conferring elasticity to the bulk structure.

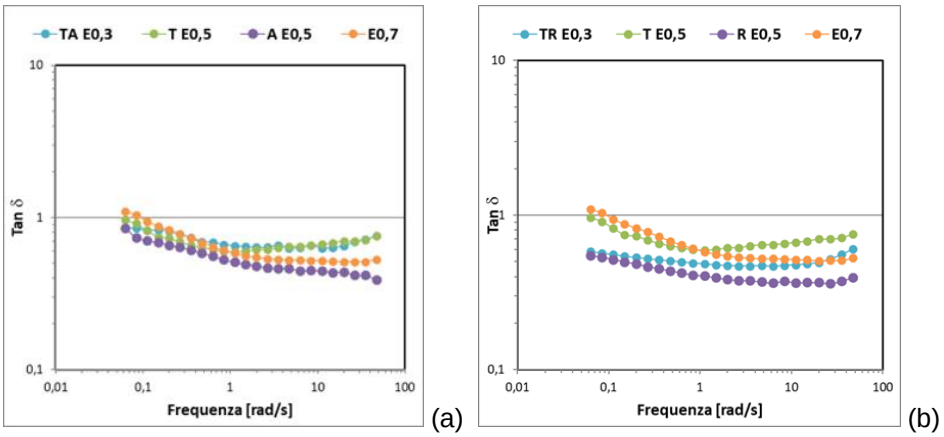


Figure 10. Tan δ values in function of frequency of the sample with the association of T and A comparing with T alone, A alone and E (a) and tan δ values of the sample with the association of T and R comparing with T alone, R alone and E (b), both at a 0.7% of total polymers concentration.

In Figure 10 the tangent delta values in function of frequency of samples prepared with TA association (Figure 10a) and TR association (Figure 10b) are reported in comparison with the binary systems. While the TA systems appear very similar to the TE binary sample, the ternary sample TR seems to be the one with the best structural properties very close to those of the RE binary gel formulation.

In all the ternary systems the presence of PFP glycerin suspension does not significantly change the characteristics of the structure (Figure 11).

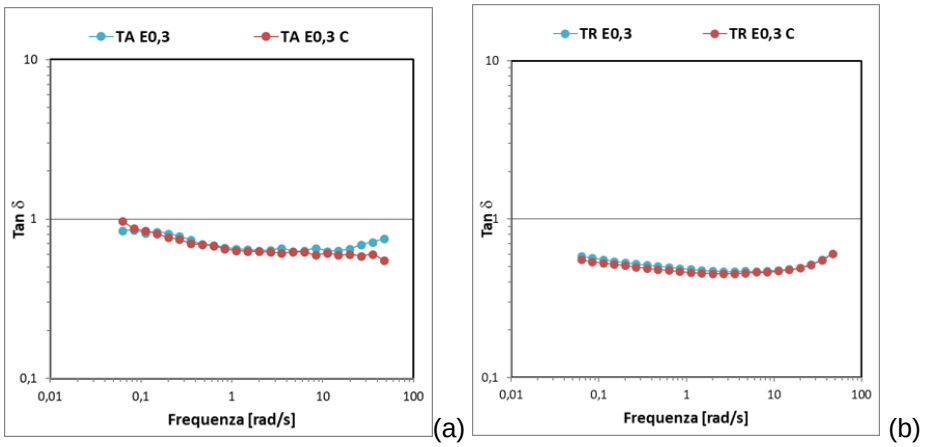


Figure 11. Ternary systems with the association of Tamarind Seed Polysaccharides (T) and *Sclerotium gum* (A), with and without the active ingredient (C) (a) and with the association of Tamarind Seed Polysaccharides (T) and *Succinoglycan gum (R)* with and without the active ingredient (b).

Discussion

Perilla frutescens phytocomplex produced by *in vitro* plant cell culture technology, is characterized by a high and standardized content of RA and anthocyanins, and can be used as a new cosmetic ingredient with sustainable and safety features related to the production process. Traditional plant extracts have extreme variability in the phytocomplex composition, and it depends on a lot of factors such as climate, soil and cultivation techniques. This variability cannot guarantee the efficacy of the extract in health care applications.

With *in-vitro* cell culture we obtained a standardized extract with high content of RA and anthocyanins free from pesticides, contaminates and residual solvents, maintaining the same biological efficacy in all batches.
PFP was tested *in vitro* for its anti-inflammatory activity and for its ability to repair skin barrier functions. The epidermis provides an important physical barrier against the environment. A dysfunction of this barrier causes increase of TEWL and penetration of external agents. Filaggrin and loricrin are important epidermal barrier proteins and their expression is correlate to the level of anti-inflammatory cytokines such as TNF-α [5]. The tight junction consists of a number of proteins. The abnormality of any tight junction associated proteins may result in the disruption of tight junction integrity and initiate disorders in the mucosa [6]. The cell bodies and the tight junctions, in fact, form the epithelial barrier that allows water and some substance of small molecular weight to pass through and restricts substance with large molecular weight to enter the deep region of the mucosa. The epithelial barrier integrity plays a critical role in the maintenance of the homeostasis in the vaginal mucosa. PFP is able to regulate both skin barrier proteins and tight junctions expression and acts with anti-inflammatory activity by inhibiting the release of inflammatory cytokines TNF-α, IL-1β and IL-6. The phytocomplex could be rationally thought as an ingredient for intimate hygiene, particularly for pre- and post-menopausal women, where the drop in circulating hormone levels, especially estrogens, represents the main trigger and consequently the vaginal epithelia display flattened epithelial surfaces with features of keratinization and the absence of papillae. Thinning of the vaginal epithelium increases susceptibility to trauma, resulting in bleeding, petechiae, and ulceration and exposes the underlying connective tissue, which is more vulnerable to inflammation or infection [7].
In the second part of this work, we studied the compatibility of PFP, presented as a powder finely dispersed in glycerin, with different classes of rheological modifiers to formulate an intimate gel. We prepared gel and gel-cream formulations with three different polymer classes: natural, semi-synthetic, and synthetic. Through the centrifuge stability test and rheological analysis, we observed that the weight of the elastic component had increased compared to the viscous one with the polymers' concentration. Rheological analyses show that in a more liquid-like structure the contribution given by PFP in structural terms to the system is seen more strongly, while in gels with weak-gel characteristics the contribution given by PFP is hardly perceptible. Moduli values between 10 and 1000 Pa, typical of a weak-gel structure, can be optimal to keep the active in suspension. *Succinoglycan gum* and *Sclerotium gum* have proved to be the most suitable natural polymers for the formulation of gels with this type of active ingredient, giving a contribution to the elastic component of the system by increasing its stability. The presence of Tamarind Seed Polysaccharides, a polymer with moisturizing and mucoadhesive properties, give to the gel and gel-cream formulations a particular contribution in the viscous component. Ternary associations of polymers, in which there is a correct balance between elastic and viscous modulus, seem to be the most suitable for obtaining products with the required characteristics of texture and stability and to better maintain PFP in suspension. The association between Tamarind Seed Polysaccharides, *Succinoglycan gum* and Esaflor HM22 seems to be the one with the best structural properties in which the active ingredient does not significantly change the characteristics of the structure but is stable in the formulation.

Conclusion

Perilla frutescens phytocomplex produced by *in vitro* plant cell culture technology, with a high and standardized association of rosmarinic acid and anthocyanins showed *in vitro* an anti-inflammatory activity and the ability to

repair skin barrier functions, by regulating skin barrier protein and tight junction expression. The polysaccharides presented greater compatibility with the active ingredient and the introduction of it into the formulation did not alter the stability of the system. *Succinoglycan gum* and *Sclerotium gum* have proved to be the most suitable natural polymers for their contribution in the elastic component of the gel, since the weak-gel structure can be the optimal one to keep the active in suspension. Tamarind Seed Polysaccharides give a contribution in the viscous modulus and have mucoadhesive properties. Ternary associations between these two polymers seem to be the most suitable for obtaining products with the required characteristics of texture and stability and to better maintain PFP in suspension.

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Conflict of Interest Statement.

None.

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