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In Vitro Cell Culture of *Rhus coriaria* L.: A Standardized Phytocomplex Rich of Gallic Acid Derivatives with Antioxidant and Skin Repair Activity

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Abstract: This study focused on the biological evaluation and chemical characterization of a new ingredient obtained by in vitro cell culture of *Rhus coriaria* L. An in vitro plant cell culture method permits to cultivate plant in a short period of time and to obtain extract with a high safety profile for the consumer, free from heavy metals, pesticides, aflatoxins, bacterial or fungal contamination. Through the selection of specific cell culture media, it was possible to obtain a *Rhus coriaria* cell line with a high content of gallic acid derivatives. The *Rhus coriaria* L. phytocomplex (RC-P), containing 7.6% *w*/*w* of acid gallic derivatives, was obtained by drying of plant cell biomass after 14 days of growth in the final selected culture medium. UPLC-ESI-MS and UPLC-DAD analysis allowed to identify numerous gallic acid derivatives, such as galloyl hexose, trigalloyl hexose and high molecular weight galloyl derivatives, and to quantify their overall content. The antioxidant activity of the RC-P was tested by DPPH assay and the wound healing activity was evaluated using a scratch wound healing test on human keratinocytes and fibroblasts. This work showed that RC-P could be a new effective cosmetic ingredient with antioxidant and skin repair activity.

Keywords: *Rhus coriaria*; phytocomplex; in vitro plant cell culture; antioxidant; wound healing

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1. Introduction

Plant cell culture technology is a technique for the growing of plant cells under strictly controlled environmental conditions [1]. The derived extracts can be easily standardized in their primary and secondary metabolites and are compliant with the safety requirements of being contamination-free and phytochemically uniform [2].

Rhus coriaria L., commonly known as sumac, is a Mediterranean species that belongs to the Anacardiaceae family and is traditionally used as a spice and flavoring agent [3]. Dried fruits, reduced to a dark red powder, with an acidic and astringent taste, is often used as spice in several Mediterranean and Middle Eastern countries. In Middle Eastern and South Asian countries, *R. coriaria* has been used, for thousands of years, as a traditional medicine for the treatment of several diseases, including cancer, liver disease, diarrhea, urinary system issues and ulcers [4]. The many therapeutic effects of *R. coriaria* could be attributed to its various biological properties, such as antioxidant and anti-inflammatory

activities. As of today, over 200 phytochemicals were isolated from *R. coriaria* and these include organic acids, phenolic acids, phenolic compounds conjugated with malic acid derivatives, flavonoids, isoflavonoids, hydrolysable tannins, anthocyanins, terpenoids, and other compounds, such as butein, iridoid, and coumarin derivatives [5].

Rhus coriaria phytocomplex (RC-P), derived from in vitro cell cultures, has a high and standardized content of gallic acid derivatives. Gallic acid is a well-known phenolic compound with great antioxidant activity, which make it suitable as an active cosmetic with antioxidant action [6]. Gallic acid inhibits the production of reactive oxygen species (ROS) and nitric oxide, proinflammatory cytokine release, and phagocytes-induced lymphocyte proliferation in human peripheral blood mononuclear cells [7]. It is also involved in various signaling pathways that regulate the wide range of biological functions, including pro- and inflammatory pathways, the NO signaling pathway, intrinsic and extrinsic pathways of apoptosis and the NF-κB signaling pathway. Recent epidemiological studies show that consumption of plant materials with antioxidant activity may decrease the risk of several diseases [8].

The skin is composed of the outer epidermis, underlying connective tissue and dermis, and functions as a barrier that protects the body from environmental stressors, such as pathogens, excessive water loss, temperature and physical stress [9]. Skin injuries, such as abrasions and incisions, may cause damage to the epidermis and/or dermal layers, requiring repair through a wound healing process consisting of four sequential phases: homeostasis, inflammation, proliferation/re-epithelialization and maturation [10]. When this well-ordered process is disrupted by factors, such as age, hyperglycemia, poor circulation, repeated trauma, continuous pressure, infections or systemic illnesses, the wound fails to close in an expected time frame, developing into a chronic wound. Proper wound healing is essential not only for the restoration of disrupted anatomical continuity and the barrier function of the skin, but also for reducing the risk of infection and further complications.

Oxidative stress by ROS production is known as a key factor for skin alteration and skin aging [11]. Polyphenols have been widely used in traditional medicines to treat several chronic skin diseases, such as psoriasis and vitiligo, and they are also known to be therapeutically beneficial in wound healing and show anti-inflammatory effects when applied topically. Gallic acid significantly improved wound healing under a hyperglucidic condition by promoting cell migration and increased expression of antioxidant genes, including catalase, SOD2, and Gpx1, which suggests the beneficial effect of gallic acid on skin aging [12]. The object of this study is to demonstrate the antioxidant and skin repair activity of a new RC-P obtained by in vitro plant cell cultures with a high content of gallic acid derivatives and its possible applications in cosmetic products.

2. Materials and Methods

2.1. Rhus coriaria Cell Line Maintenance and Growth

Cell culture of *Rhus coriaria L.* was obtained from plants bought and certified from the nursery plant "Le Georgiche", Brescia, Italy. The botanical origin of *R. coriaria* was confirmed by fingerprint analysis made from Parco Tecnologico Padano, Lodi, Italy [13].

Young leaves 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 min, 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 2–4 min and, finally, at least five washes with sterile distilled water. The leaves were cut into small pieces (explants) of sub-centimetric dimensions (0.1–0.5 cm). The fragments of plant tissue were deposited in several Petri dishes containing solidified Gamborg B5 Medium [14] supplemented with 20 g/L sucrose (Sudzucker AG, Manheim, Germany), and different phytohormone compositions. Different combination of Kinetin (K) (Duchefa), naphtalenacetic acid (NAA) (Duchefa), indolacetic acid (IAA) (Duchefa), Picloram (PIC) (Duchefa), 6-benzylaminopurine (BAP)

(Duchefa), indole-3-butyric acid (IBA) (Duchefa) and 2,4-dichlorophenoxy acetic acid (2.4D) (Duchefa) have been tested in a concentration range between 0.1 to 3 mg/L. The pH was adjusted to 6.5 before autoclaving. Petri dishes containing explants were incubated at 25 ± 2 °C in the dark condition. The medium that most stimulated the callogenesis of explants contained 20 g/L sucrose, 1 mg/l of NAA, 1 mg/L of IAA, 0.1 mg/L of K and 0.8% (*w*/*v*) of plant agar, final pH 6.5 (RC Medium). Calli of *R. coriaria* grown on solid RC Medium were subjected to subculture for at least 4 months until they become friable and homogeneous, with a constant growth rate. The suspension cultures were obtained transferring a part of selected calli (10% *w*/*v*) in 1 L Erlenmeyer flasks containing 250 mL of culture liquid Medium (RC Medium without Plant Agar). The suspension cultures were maintained at 25 °C in the dark in constant agitation on a rotary shaker at 110 rpm, and every 7 days of fermentation, they were subjected to a liquid subculture. The volume of biomass was increased by subculture on 3 L flasks containing 1 L of fresh RC liquid Medium. The amount of suspension culture inoculated into the liquid medium was equal to 10% *v*/*v*. To increase the content of gallic acid and his derivatives, after 7 days of fermentation, the suspensions culture was inoculated in a bioreactor of 5 L volume containing 3 L of liquid medium with a different qualitative-quantitative composition with respect to the RC liquid medium. Several combinations of salts, nutrients and phytohormones have been tested, and the composition that allowed us to maximize the content of the gallic acid and his derivatives was Gamborg B5 with the addition of 40 g/L of sucrose, 0.5 mg/L of NAA, 0.2 mg/L of IAA and 0.02 mg/L of K, final pH 6.5 (RC final liquid Medium). The final suspension culture was grown in RC final liquid medium for a culture cycle of 14 days.

2.2. Phytocomplex Preparation from Rhus coriaria Cell Culture

After 14 days of growth, at 25 °C in the dark condition, the *R. coriaria* cell suspensions were filtered by 50 µm mesh filter, and the medium culture 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 homogenated with Ultraturrax at 15,000 rpm for 20 min. The biomass of homogenated cells was dried to obtain a powder of RC-P.

2.3. UPLC-ESI-MS Analysis

UPLC-ESI-MS analysis were used only for the identification of the secondary metabolites of the RC-P. For untargeted metabolomics analysis, cells from three independent culture batches of *R. coriaria* were collected and nitrogen frozen, powdered and then extracted with six volumes of LC-MS grade 100% methanol (W/V) for 15 min in an ultrasonic bath in ice (SOLTEC, Milano, Italy). The extracts were collected by centrifugation (14,000× *g* for 10 min), diluted 1/10 with LC-MS grade water (V/V), filtered through Minisart RC4 filters with 0.2 mm pores (Sartorius, Göttingen, Germany) and injected into the UPLC device (1 mL/sample). Untargeted metabolomics analysis and data processing were performed as described by Commisso et al. [15], using an ACQUITY I CLASS UPLC system (Waters, Milford, MA, USA) that was connected to a Xevo G2-XSqTOF mass spectrometer (Waters) featuring an electrospray ionization (ESI) source operating in negative ionization. Briefly, extracts were injected into a BEH C18 column (2.1 mm × 100 mm, 1.7 µm) kept at 30 °C and the mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B). The initial conditions were 99% A and 1% B, and the following elution profile was applied: 0–1 min, 1% B; 1–10 min, 1–40% B; 10–13.50 min, 40–70% B; 13.50–14.00 min, 70–99% B; 14.00–16.00 min, 99% B; 16.00–16.10 min, 99% A and 1% B (initial conditions). Subsequently, the system was equilibrated in 99% A and the elution was complete after 20 min. The flow rate was set to 0.350 mL/min. Samples were kept at 8 °C and randomized. The annotation of metabolites was performed by automatic online search of public databases (MassBank, PlantCyc, Plant Metabolic Network and Human Metabolome Database, Metlin), by comparing m/z ratios, isotope similarities and fragmentation patterns (ms/ms), using the individual compound fragmentation extrapolated by Progenesis QI software, and using and an in-house library of authentic standards. All the identifications were manually checked.

2.4. UPLC-DAD Analysis

UPLC-DAD analysis was used for the quantification of the total of gallic acid derivatives in the RC-P. Powder of the RC-P (100 mg) were weighed into a 15 mL test tube, and 30 volumes of ethanol (Honeywell) and water 60:40 (v/v) were added. The suspension was mixed for 30 s with a vortex mixer and sonicated for 15 min in an ice bath; finally, it was centrifuged at 4000 rpm for 15 min at 6 °C. At the end of centrifugation, the supernatant was recovered. A volume of 15 mL of supernatant were transferred into a new test tube of 15 mL 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 the RC-P were extracted and measured.

The chromatography system used for the quantification of gallic acid derivatives consist of an Acquity UPLC BEH C18 1.7 µm column, size 2.1 mm × 100 mm, coupled to an Acquity UPLC BEH C18 1.7 µm VanGuard Pre-column 3/Pk, size 2.1 mm × 5 mm. The platform used for the UPLC-DAD (Ultra Performance Liquid Chromatography-Diode Array Detection) analysis comprised a UPLC system (Waters Corporation, Milford, MA 01757, USA) 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 (Photo Diode Array) eλ diode array detector. The data were acquired and analyzed using Empower 3 (Waters) software. 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.

Table 1. Elution of the molecules in UPLC-DAD (Ultra Performance Liquid Chromatography-Diode Array Detection) analysis conducted by alternating gradient and isocratic phases and used for the quantification of gallic acid derivatives.

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

For quantification of the gallic acid derivatives, the chromatogram obtained at the wavelength of 270 nm was used. The gallic acid and his derivatives were quantified with the calibration curve of the authentic commercial standard of gallic acid (CAS 149-91-7; purity ≥ 99% Extrasynthese ID:0728/0 Batch:06). The data analysis was carried out with Empower 3 software.

2.5. Cell Culture

The human keratinocyte cells (HaCaT) and human foreskin fibroblast cells (HFF-1) were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in complete medium containing DMEM (Merck KGaA, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Merck KGaA, Germany), 1% penicillin/streptomycin solution (Merck KGaA, Germany) and 1% of L-glutammine (Merck KGaA, Germany) and passed by trypsinization. All treatment experiments were performed in reduced FBS (5%) condition.

2.6. Antiradical Capacity: DPPH Assay

The antiradical capacity of RC-P was tested through a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, performing the method previously optimized by Biagi et al. [16]. Tested samples were dissolved in ethanol 96% v/v. Different sample concentrations (range 6.25–0.19 mg/mL) were mixed with DPPH (1:19) and, after incubation for 30 min at rt in the dark, the absorbance was read at 515 nm using a Perkin-Elmer Victor Nivo3s plate reader. IC_{50} values were calculated using linear regression analysis. Three independent replicates were performed.

2.7. Scratch Wound Healing Assay

Scratch wound healing assay on human keratinocytes and fibroblasts was used to demonstrate the healing activity. Briefly, HaCaT cells (5×10^4) and HFF-1 cells (5×10^4) were seeded into six-well cell culture plates and allowed to grow to 70–80% confluence as a monolayer. The monolayer was gently scratched across the center of the well with a sterile one-mL pipette tip. A second scratch was performed in a perpendicular way to the first, creating a cross in each well. After scratching, the medium was removed, and the wells were washed twice in PBS (Merck KGaA, Germany) solution. Fresh medium containing treatments was added to each well, and cells were grown for 24 h. Images were obtained from the same fields immediately after scratching (t0) and after 6 h and 24 h using a Leica DMIL microscope and analyzed using ImageJ software by manually selecting the wound region and recording the total area. The experiments were conducted in triplicate, and two fields were analyzed for each replicate (n = 6). Untreated scratched cells represented the control.

The percentage of wound closure was calculated using the following formula:

$$\% \text{ Wound closure} = [(\text{Wound area t0} - \text{Wound area t})/\text{Wound area t0}] \times 100$$

The scratch wound healing assay was also performed in a model of reduced cell proliferation induced by 6α-methylprednisolone (MET, 1 mg/mL), thus, mimicking an unbalanced cell turnover condition, similar to that of aged skin.

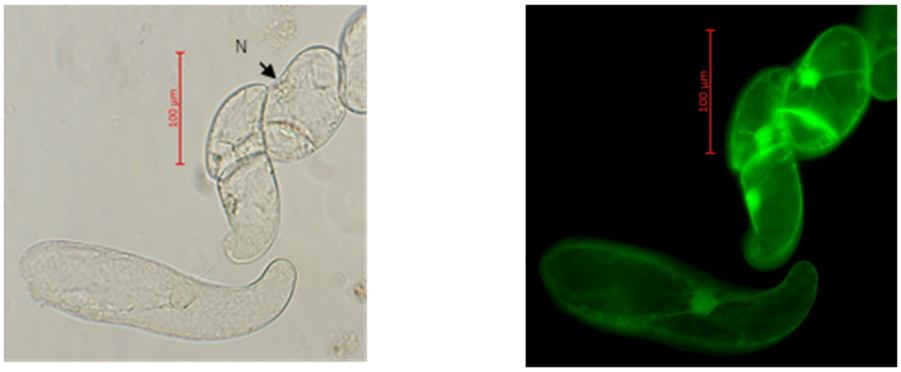
2.8. TGF-β and EGF Dosage

HaCat cells (1 × 105 cells/well) were seeded into 24-well plates and cultured for 24 h. Cells were treated for 24 h with 100 µg/mL of RC-P. TGF-β and EGF production was evaluated in cell medium according to the manufacturer's instruction by non-competitive sandwich ELISA (Invitrogen) dosages, as previously reported [17]. Absorbance was measured at 450 nm in the plate reader. Samples were assayed in duplicate. Dosages were performed in three independent experiments.

3. Results

3.1. Standardized Rhus coriaria Phytocomplex

A cell line stable both in solid and liquid RC Medium was obtained starting from a few young leaves of *R. coriaria*. It was characterized by smalls elongated cells with lateral nucleus and rich in cytoplasmatic strands. Figure 1 shows the cells in brightfield mode (a) and after staining with fluorescein diacetate (b).



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

Figure 1. Microscopic images of *Rhus coriaria* cells taken using AXIO-Imager A2 optical microscope (ZEISS) at a magnification of 20×. (**a**) In the brightfield mode; N, nucleus. (**b**) Cells after staining with fluorescein diacetate. Scale bar: 100 µm.

The total content of gallic acid derivatives, expressed as equivalent of gallic acid, was increased and optimized using RC final liquid Medium with a specific quantitative composition: higher content of sucrose (40 g/L) and lower concentration of phytohormones (0.5 mg/L of NAA, 0.2 mg/L of IAA and 0.02 mg/L of K).

3.2. UPLC-ESI-MS Analysis

RC-P, prepared by suspension cell cultures, was analyzed by UPLC-ESI-MS, which provided the presence of several gallic acid derivatives. The main metabolites in the chromatogram of one representative sample are reported in Figure 2 and in Table 2.

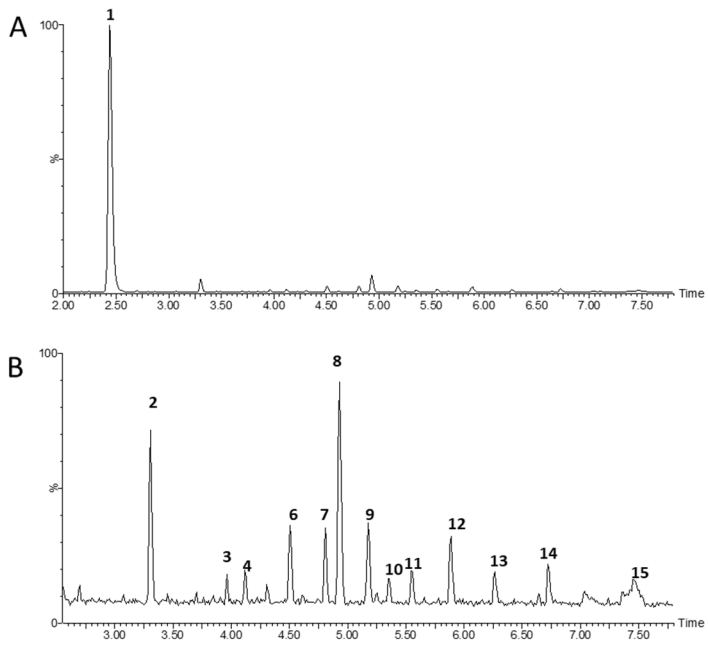


Figure 2. UPLC-MS chromatogram of one representative *R. coriaria* sample, which shows that the presence of gallic acid derivatives (**B**) is a detail of (**A**).

Table 2. Main metabolites of *Rhus coriaria* samples. The identification numbers (id) are the same as in Figure 2; 14 and 15 were not identified. Galloyl hexose was the more represented metabolite. Numerous other derivatives of gallic acid (galloyl hexose, trigalloyl hexose and high molecular weight galloyl derivatives), hydroxycinnamic acid derivatives, catechin, procyanidins and flavonoids were also detected. rt = retention time; ui = unidentified; m/z = mass-to-charge ratio * confirmed by comparison with authentic standard; ** not confirmed by fragmentation.

id	rt	m/z(-)	Fragments	Putative Identification
1	2.44	331.066	169.014	Gallic acid hexose
2	3.3	399.148	171.9463; 263.0213	ui
3	3.96	413.165	-	ui
4	4.117	371.098	161,0238; 163,039	Cumaric acid hexose
5	4.3	341.085	-	Caffeic acid hexose
6	4.5	387.131	-	ui
7	4.8	289.071	*	Catechim *
8	4.9	325.09	163.036	Cumaric acid hexose
9	5.179	325.094	-	Cumaric acid hexose **
10	5.35	635.092	287.055	Trigalloyl hexose **
11	5.55	461.165	-	ui
12	5.889	447.094	285.0394	Tetrahydroxyflavone hexoside
13	6.264	447.09	163.0031; 227.0709; 245.0804; 255.0296; 285.0394	Tetrahydroxyflavone hexoside

The identification numbers (id) in Table 2 are the same as in Figure 2.

Galloyl hexose was the more represented metabolite. Numerous other derivatives of gallic acid, hydroxycinnamic acid derivatives, catechin, procyanidins and flavonoids were also detected. The complete list of putatively identified metabolites can be found in the Supplementary Material Table S1.

3.3. UPLC-DAD Analysis of Gallic Acid Derivatives

The quantification of gallic acid derivatives in RC-P was carried out by using an UPLC-DAD method validated in terms of linearity, precision and limit of detection (LOD)/limit of quantification (LOQ).

A calibration curve was obtained by the external standard method, using six different concentrations of the commercial standard with two injections per amount. The peak areas measured at 270 nm wavelength were plotted against the known concentrations of the standard solutions to establish the calibration equation; linear regression equation was calculated via the least squares method. This method showed an excellent linearity at concentrations from 1.7 to 346 μg/mL; the correlation coefficient (R2) of the regression equation presented was 0.9999. To validate the method against criteria for precision, the gallic acid standard was analyzed at three different concentrations (136.0, 228.0 and 319 μg/mL) to verify the repeatability. Three arbitrary concentrations of gallic acid standard were selected and the residual standard deviation (RSD, %) were calculated from the results of the repeated measurements for each concentration. The results showed that RSDs for gallic acid at concentrations of 136.0, 228.0 and 319 μg/mL were 0.71, 0.59 and 0.11%, respectively.

These results indicate that the precision criterion for the determination of gallic acid concentration is acceptable because the RSD is below 2%, which corresponds to the criterion recommended by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [18]. The LOQ (Limit of Quantification)/LOD (Limit of Detection) values for gallic acid were 1.5 µg/mL and 4.9 µg/mL, respectively. The calibration curve of gallic acid can be found in the Figure S1 and the data in the Tables S2 and S3 of the Supplementary Material.

Then, the chromatographic areas at 270 nm wavelength of the gallic acid derivatives present in the RC-P extract (Figure S2 of Supplementary Material) were measured and compare with the calibration curve. The total gallic acid derivatives were expressed as the equivalent of gallic acid.

The content of gallic acid derivatives, identified by their characteristic UV spectrum with λmax at 270 nm and expressed as the equivalent of gallic acid in the *R. coriaria* suspension culture after 14 days of growth in RC final liquid medium was 2.5 ± 0.2 g/L, while in the RC-P this was 7.6 ± 0.1% *w*/*w*.

3.4. Scratch Wound Healing Assay, Evaluation of EGF and TGF-β Levels and DPPH Assay

The ability of the in vitro model to simulate keratinocytes wound healing was confirmed by the time-dependent increased wound healing of the untreated control after 6 and 24 h. RC-P at the concentration of 0.1 mg/mL significantly increased the wound closure rate by more than 40%, compared to the untreated control (Figure 3a). This effect was still significant after 24 h (Figure 3b). No effects were observed using 1 mg/mL or 0.01 mg/mL. Another confirmation of the ability of our in vitro model to simulate keratinocytes physiologic behavior was obtained by using MET 1 mg/mL to mimic a reduced cell proliferation condition. Indeed, the wound healing of the MET-treated cells was significantly reduced, compared to the untreated control, at both 6 and 24 h (−73% and −39%, respectively). Interestingly, the wound healing activity of RC-P was more pronounced in this condition, and evident at each tested concentration after both 6 and 24 h, with 0.1 mg/mL remaining the most active concentration.

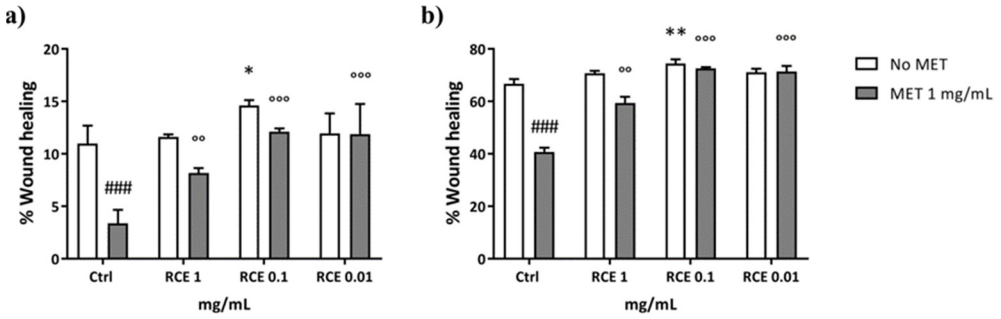


Figure 3. Wound healing activity on keratinocytes of RCE (RC-P extracts) after (**a**) 6 and (**b**) 24 h of treatment, in the absence and presence of MET (6α-methylprednisolone). Two-way ANOVA, followed by Sidak post-hoc ### $p < 0.001$ vs. Ctrl; * $p < 0.05$ vs. Ctrl; ** $p < 0.01$ vs. Ctrl; °° $p < 0.01$ vs. MET; °°° $p < 0.001$ vs. MET.

On human fibroblasts, the scratch wound healing assay was performed and tested at a lower concentration of RC-P, i.e., 0.001 mg/mL, excluding, on the other hand, 1 mg/mL, which in our first replicates was ineffective. RC-P at a concentration of 0.01 mg/mL showed the highest activity in reducing wound area after 24 h of treatment compared to untreated cells (Figure 3). No significant effects were observed using 1, 0.1 and 0.001 mg/mL concentrations. Following MET (1 mg/mL) stimulation, the fibroblasts ability to close the wound was reduced by about 40% after 24 h compared to control cells. In these conditions, RC-P at the concentrations of 0.1 and 0.001 mg/mL was able to significantly induce the wound healing after 24 h of treatment (Figure 4). Although not statistically significant, RC-P 0.01 mg/mL showed the same trend in reducing the wound area.

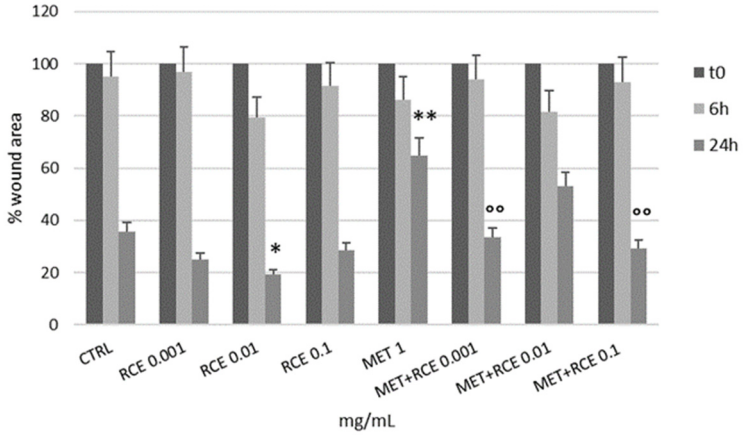


Figure 4. Wound healing activity on fibroblasts of RCE (RC-P extracts) after 6 and 24 h of treatment, in the absence and presence of MET (6α-methylprednisolone). Two-way ANOVA, followed by Tukey post-hoc: * $p < 0.05$ vs. ctrl; ** $p < 0.01$ vs. ctrl; °° $p < 0.01$ vs. MET.

To investigate the possible molecular mechanisms underlying the repairing activity of RC-P, the expression levels of EGF and TGF-β, two of the main growth factors contributing to the proliferation and migration processes were measured by ELISA assay in keratinocytes treated with 0.1 mg/mL of RC-P for 24 h. The expression levels of EGF and TGF-β were not modified by the treatment with RC-P, excluding the involvement of this mediators in the mechanism of action of the pythocomplex. With the aim of evaluating the antiradicalic activity of RC-P, we performed a DPPH assay that showed the high scavenger capacity with IC_{50} equal to 16.01 ± 0.16 μg/mL.

4. Discussion

RC-P with a high content of gallic acid derivatives, expressed as the equivalent of gallic acid (7.6% *w*/*w*), produced by in vitro plant cell culture technology, is a new cosmetic ingredient with sustainable and safety features related to the production process [19]. The high content of gallic acid derivatives in the *R. coriaria* selected cell line is due to the high plasticity of the de-differentiated cells cultured in optimized medium and under controlled conditions. Plants are sessile organisms that have a remarkable developmental plasticity, which ensures their optimal adaptation to environmental stress. Plant cell totipotency is an extreme example of such plasticity, whereby somatic cells have the potential to form plants via direct shoot or somatic embryogenesis in response to various exogenous and/or endogenous signals [20]. The ability of somatic plant cells to change their development fate and to regenerate new tissues, organs and the whole plant is a widely known phenomenon [21,22]. The switch from a somatic to a totipotent or pluripotent cell state involves cellular reprogramming, including chromatin reorganization and changes in gene expression patterns and in the cell wall composition and architecture [23–25].

The cell plasticity of the in vitro plant cell culture allows to induce rapid stimuli to the biosynthesis of secondary metabolites by modifying the final liquid medium composition. The RC final liquid medium was optimized by studying different qualitative-quantitative combinations of growth hormones and sucrose as described in the materials and methods of this work. Higher content of sucrose and lower concentration of growth hormones (in comparison to the quantity present in the RC liquid medium) induced the biosynthesis of a high content of gallic acid derivatives in *R. coriaria* suspension cultures. Traditional plant extracts have extreme variability in their 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 gallic acid derivatives free from pesticides, contaminates and residual solvents, maintaining the same biological efficacy in all batches [26]. Gallic acid and other antioxidant compounds improved wound healing by decreasing wound

area, increasing fibroblast cells, reducing inflammatory cell infiltration, and decreasing inflammation, acting on redox balance and oxidative stress in healing process [27,28].

The effect of different *R. coriaria* fruit extracts on skin wound healing have been reported. Gabr and Alghadir showed that the topical application of an aqueous extract obtained from *R. coriaria* fruits, containing hydrolyzable tannins, gallic acid, quercetin and myricetin, was able to facilitate the wound healing process of infected and non-infected rat skin, by modulating the inflammatory response, through the increase of matrix metalloproteinase 8 and myeloperoxidase activity [29]. In a different in vivo model in rats, the dressing of wounds with a hydrogel containing *R. coriaria* fruits ethanolic extract was able to enhance the wound healing process, by stimulating the production of hydroxyproline and nitric oxide [30].

However, to the best of our knowledge, the effect of samples derived from in vitro cell cultures has never been demonstrated previously. For this reason, in this work, the biological activities of RC-P, obtained by a selected cell line, were tested in an in vitro model of wound healing using human keratinocytes and fibroblast cell lines. RC-P at a concentration of 0.1 mg/mL was able to induce significant keratinocytes migration and wound closure after 6 h and 24 h of treatment. Interestingly, the wound healing capacity was reduced at higher concentrations. This behavior has been already observed using other polyphenol-enriched products and is related to the ability of high concentration of polyphenols to reduce the production of pro-inflammatory cytokines and interact with cells metabolism and proliferation, thus reducing wound healing [17,31]. Under stress conditions, such as the administration of corticosteroids, the physiological ability of epidermis cells to repair damage induced by abrasions or wounds was impaired. RC-P showed the best activity under these conditions by stimulating keratinocytes to close the wound both after 6 and 24 h. The efficacy of RC-P in a model of reduced wound healing is consistent with the increased wound contraction observed by Alsarayreh and co-authors using a methanolic extract of *R. coriaria* fruits in diabetic and non-diabetic rats, which was related to the reduction of IL-6 and the increase of IL-10, hydroxyproline and collagen expression [32]. The activity of RC-P on the fibroblast component of epidermis has proved to be as effective as on keratinocytes but only after 24 h of treatment in both basal and stress conditions. The reason of a delay in the fibroblasts response could be attributed to the slower migration and proliferation ability, and slower metabolic activity of this cell line. The analysis of the main growth factor, EGF and TGF-β, implicated in cell proliferation and migration properties showed that RC-P activity did not alter the levels of these factors in keratinocytes. Although the activity of RC-P did not involve EGF and TGF-β growth factors, its phytocomplex showed a high antioxidant capacity that could be at the base of its biological function. Indeed, several bioactive antioxidants were found to be able to accelerate skin wound healing. In particular, the wound healing capacity of gallic acid, the main constituent of RC-P, has been investigated *in vitro*, in animal models and in humans (*ex vivo*) [28], and was related to the reduction of ROS production [33] and the upregulation of endogenous antioxidant enzymes, such as superoxide dismutase 2, catalase and glutathione peroxidase 1, together with the activation of focal adhesion kinases, c-Jun N-terminal kinases and extracellular signal-regulated kinases [12].

Together with the data already available in the literature, our results suggest that the mechanism of action of RC-P in wound healing is mainly due to its antioxidant and anti-inflammatory activity, and is not mediated by the modulation of EGF and TGF-β.

5. Conclusions

RC-P derived from in vitro cell cultures has a high and standardized content of gallic acid derivatives, expressed as equivalent of gallic acid (7.6% *w*/*w*), a well-known phenolic compound with great antioxidant activity. The high content of gallic acid derivatives in this cell line is due to the high plasticity of the de-differentiated cells cultured in optimized medium and under controlled conditions. RC-P, tested on an in vitro wound-healing model with human keratinocytes and fibroblast cell lines, demonstrated the ability to induce

significant keratinocytes migration and wound closure after 6 h and 24 h of treatment, at a concentration of 0.1 mg/mL. RC-P showed the best activity under stress condition on keratinocytes and a high antioxidant capacity. It is a new ingredient for cosmetic products with antioxidant and skin repair activity with sustainable and safety features related to the production process.

6. Patents

Pressi, G.; Bertaiola, O.; et al. Phytocomplex and Extract of a Meristematic Cell Line of *Rhus coriaria*. Patent ITA 102020000028136/ PCT IB2021/057646.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics9010012/s1, Table S1: *Rhus coriaria* putatively identified metabolites by means of UPLC-ESI-MS. rt (retention time), detected m/z (mass-to-charge ratio), calculated m/z, mass error (in PPM), elemental formula are reported; Figure S1: Calibration curve of gallic acid; values are mean of two independent measures. The linear regression equation is reported (R^2 = 0.9999), Table S2. Results of linearity regression, correlation coefficient, LOD and LOQ for gallic acid standard. Table S3: Intra-day variability of gallic acid standard, Figure S2: Chromatographic profile of RC-P extract at the wavelength of 270 nm, using the UPLC-DAD analysis, to estimate the content of gallic acid derivatives. Galloyl derivatives are identified by their characteristic spectrum with λmax at 270 nm.

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