A new technological approach to improve the efficacy of a traditional herbal medicinal product in wound healing

Maria Letizia Manca, Marco Zaru, Gianluigi Bacchetta, Teobaldo Biggio, Nadia Cappai, Alba Cabras, Angela Maria Falchi, Maria Manconi, Anna Maria Fadda

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Sardinian folk medicine has given rise to a renewed interest in treatment of different diseases, particularly skin burns and inflammatory disorders. Rubus ulmifolius Schott shoots have been used to treat burns of different degree. During this work, a new and more effective extraction method was developed using olive oil at room temperature for 10 days. The R. ulmifolius oleolite components were mainly represented by polyphenolic compounds and tannins. To obtain a suitable topical formulation, the Rubus oleolite was incorporated in an ointment composed of anhydrous lanolin, white wax, olive oil, and Europhoe oil unsaponifiables. Both R. ulmifolius oleolite and ointment were able to scavenge DPPH free radicals in vitro in a way very similar to quercetin, used as reference. In addition, R. ulmifolius ointments were biocompatible in vitro on 3T3 fibroblasts and did not cause any malformation or cell degeneration. Moreover, in vivo studies on Sprague-Dawley rats confirmed the ability of Rubus ointment to improve skin regeneration after burns.

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1. Introduction

Partial and full thickness burns are acute wounds, which result in local and deeper tissue injury and loss. Wound healing is a complex process involving many physiological actions and responses, requiring the collaborative effects of different tissues and cells. Actually, free radicals are generated at the site of injury and hamper the healing process. The use of antioxidants has been shown to promote wound healing: several natural compounds like flavonoids, triterpenes, alkaloids and polyphenolic biomolecules might positively influence one or more phases of the healing process. It is well known that plants are rich in these antioxidant and anti-inflammatory compounds. Thus, they represent an inestimable source of molecules capable of treating diseases involving an overproduction of oxygen reactive species and inflammatory mediators, such it happens with as mucosa, epithelia and skin lesions. Indeed, wound healing properties of plants, in most cases, are associated with their significant antioxidant activities (Süntr et al., 2012). Several plant extracts or their antioxidant enriched fractions have been extensively investigated for these therapeutic uses: as an example quercetin, a phytolflavonoid widespread in fruits and vegetables, is one of the most potent and commonly used antioxidant (Ahmad et al., 2013). Tocopherol is another naturally occurring powerful antioxidant present in several foods. The unsaponifiable fractions of natural plant oils are particularly rich in these and others active components and have been tested for the treatment of several diseases involving accumulation of oxidative species (Djerrou et al., 2010). For instance, the well known antiatherosclerotic, antioxidant, and anti-inflammatory effects of extra virgin olive oil have been shown to be especially due to the presence of tocoferol and others phenolic compounds mostly contained in its unsaponifiable fraction (Waterman and Lockwood, 2007; Miles et al., 2005; Visioli et al., 2002).

Among others, Rubus ulmifolius Schott species are usually rich in quercetin, polyphenols and carotenoids and have been used for wounds healing, infected insect bites, and pimpls in folk medicine. In particular, R. ulmifolius was extensively used against inflammatory disorders and skin wound healing in traditional Sardinian
medicine, by treating the plant shoots in olive oil to extract all the active components (Süntar et al., 2011; Deighton et al., 2000), working in "drastic conditions".

In the present study, according to ethnophytomedicine, we associated the antioxidant ability of the unsaponifiable fraction of olive oil with that of R. ulmifolius shoots to obtain a potent antioxidant and anti-inflammatory oleolite (Zaru et al., 2013). The simultaneous presence of different antioxidants is important because they may act in different ways (i.e. binding metal ions, scavenging radicals and decomposing peroxides) and, often, involving more than one mechanism may result in a synergic and improved final formulation efficacy (Süntar et al., 2012; Akkol et al., 2011).

Aimed to select an appropriate preparation method to obtain a suitable formulation, the R. ulmifolius shoot biomolecules were extracted in olive oil at 25 °C or at 150 °C and the obtained extract components were identified, quantified and compared. Finally, to potentiate the polyphenol fraction, Olea europaea oil unsaponifiables were added. A natural based formulation was developed gelling the oil in lanolin/wax ointment to provide more efficient wound healing in situ activity. Here, we report the R. ulmifolius oleolite composition and utility of its ointment for the active healing of topical burn wounds in vivo. Moreover, in vivo skin tolerability and in vitro cytotoxicity were evaluated.

2. Experimental

2.1. Materials

Anhydrous lanolin (Ph Eur), white beeswax (Ph Eur), purified olive oil (Ph Eur) and Olea europaea oil unsaponifiables were obtained from Galeno srl (Carmignano, PO). Phosphate buffer solution (PBS, pH 7), quercetin (QUE), caffeic acid, kaempferol and all other products and solvents of analytical grade were purchased from Sigma–Aldrich (Milan, Italy). Connettivinò® was purchased in a pharmacy (Fidia Farmaceutici s.p.a., Abano Terme (PD) Italy).

2.2. Plant extraction and identification of main components

R. ulmifolius shoots were collected in southern Sardinia (Grosagutu, Assemini 39.29° latitude and 8.99° longitude) during the flowering season.

Collected shoots were identified by Centro Conservazione Biodiversità (CCB), University of Cagliari. Subsequently R. ulmifolius shoots were taken to the Germoplasm Bank of Sardinia (BC-SAR), here the shoots were processed, characterized and selected before conserving them in the Bank (Bacchetta et al., 2004; Pabst et al., 2000). Then within 12 h from collection, R. ulmifolius shoots were dried at 40 °C for a period of 24 h as determined by quantifying the loss of water. Oleolite preparation was carried out dispersing dried and pulverized shoots (7.5 g) in 75 g of olive oil (Ph Eur) at 150 °C for 2 min (hot method) or alternatively at 25 °C for 10 days (room temperature method). Afterwards, the obtained oleolite was filtered and stored.

The different components of oleolite were separated and identified by high performance liquid chromatography (HPLC) using a chromatograph Shimadzu LC 2010 equipped with a photo-diode array detector and a column Symmetry RP18 (Water, 5 μm, 4.6 mm x 250 mm). Two different mobile phases were used: the first one (A) was a mixture of water/acetic acid (97/3, v/v) and the second one (B) was a mixture of acetonitrile/methanol (50/50, v/v), delivered according to a constant gradient (increasing 2% B/each minute), and a flow rate of 1 ml/min. Detection wavelength was 290 nm, injection volume was 100 μl and the limit of quantification ranged from 0.1 to 0.01 μg. Five different fractions were obtained and their main components were then identified by HPLC–MS (mass spectrometry) using a Thermo LTX XL Linear Ion Trap Mass Spectrometer. The mobile phase was a mixture of water/methanol (90/10, v/v). Identification and quantification of the main components were carried out using reference standards.

2.3. Loss on drying

The loss on drying test was performed according to Ph. Eur., to evaluate the loss on mass expressed as per cent m/m. The R. ulmifolius shoots were placed in a weighing bottle previously dried and dried in an oven at 40 ± 5 °C to constant mass (24 h).

2.4. Ointment preparation

The appropriate amount of lanolin and white wax were melted (~70 °C) and then mixed with the oleolite and unsaponifiable fraction of olive oil; sodium benzoate and butylated hydroxyanisole were added as preservative and antioxidant respectively. The dispersion was homogenized initially at 2000 rpm for about 10 min during which water phase previously warmed at about 70 °C, was also added. The homogenization process continued at 3000/3500 rpm for further 30 min; the dispersion was maintained under gentle stirring until the temperature decreased reaching 37 °C. Finally, the ointment was transferred into a suitable vessel and stored for all subsequent studies.

2.5. In vitro DPPH assay

The antioxidant activity of R. ulmifolius ointment was tested by measuring its ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) in comparison with quercetin methanolic solution scavenger ability. Each sample was dissolved (1:50 v/v) in DPPH methanol solution (25 μM). DPPH methanolic solution at the same dilution (1:50 v/v) was used as control. All the samples were stored at room temperature for 30 min, in the dark. Then, the absorbance was measured at 517 nm against blank. All the experiments were performed in triplicate. The percent antioxidant activity (or free radical scavenging activity) was calculated according to the following formula (Caddeo et al., 2013; Manca et al., 2014): antioxidant activity (%) = [(ABS DPPH − ABS sample)/ABS DPPH] × 100.

2.6. Cytotoxicity test by direct contact

Mammal fibroblasts BalbC 3T3 were used to perform cytotoxicity tests by direct contact. Cells were incubated at 37 °C in 5% CO2 humidified atmosphere, and Dulbecco’s Modification of Eagle’s Medium (DMEM), supplemented with calf bovine serum (FBS), penicillin, streptomycin and fungizone was used as growth medium. Cells were grown as subconfluent monolayer for 24 h in 12 well plates. R. ulmifolius ointment (50 μl) was deposed on filter paper (3 wells), only filter paper was used as negative control (3 wells), latex film as the positive control (3 wells) and, finally, medium without cells (3 wells) was used as blank. Plates were incubated for 8, 24, 48 and 72 h; successively cells were observed using an inverted microscope. During the experiment, biological reactions (cell degeneration and malformations) were evaluated according to ISO1 0993-5:2009, following a 0–4 scale (0 indicates none reactivity and no detectable zone around or under specimen; 1 indicates slight reactivity and some malformed or degenerated cells under specimen; 2 indicates mild reactivity and zone limited to area under specimen; 3 indicates moderate reactivity and zone extending specimen size up to 1 cm; 4 indicates severe reactivity and zone extending farther than 1 cm beyond specimen).

Quantitative evaluation of cytotoxic effect of formulation was performed using the natural red uptake method that allows to measure cell viability using their capacity to incorporate and to bind...
a cellular vitality dye, the Neutral Red. After 24 h, each well was treated with Neutral Red Medium (2 ml) for 3 h, washed with PBS and finally treated with destobor solution (2 ml). The plate was gently stirred for 10 min and the optical density (OD) was read spectrophotometrically at 540 nm using a Microplate Reader El800-Pc (BioTek Instrument, US). Vitality results are shown as a percentage of the negative control:

$$\text{Vitality}\% = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{negative control}} - \text{OD}_{\text{blank}}} \times 100.$$ 

2.7. In vivo hypersensitivity test

The study was performed according to standards of Good Laboratory Practices and was approved by Eurofin Biobal Srl (certification no. 121/2010 and provisional certification 2012). Delayed hypersensitivity test (GPMT test) of *R. ulmifolius* ointment was carried out according to ISO 10993-10:2010. Eighteen male, albino guinea pigs (300–500 g) were used, 10 of which were treated with the test samples, 5 were used as a control group and 3 for preliminary test. The animals were caged in group of five; the housing room was lighted with fluorescent lamps and maintained with light and dark cycles (12 h each). Room temperature (20 ± 3 °C) and humidity (>30%, <70%) were regulated and monitored daily.

Preliminarily, the highest concentration causing mild to moderate erythema, without any other adverse effects, was selected for the topical induction phase (3 animals). Then, the highest concentration that produced no erythema was selected for the challenge phase (3 animals).

Occlusive patches with undiluted sample (0.5 ml) and diluted sample (75% and 50% in sodium chloride) were applied for 24 h onto the guinea pig back (n = 3).

In the induction phase, 10 animals were treated with samples and 5 were used as controls. At day 0, guinea pigs were treated by 3 intradermal injections (0.1 ml of 3 different samples): 1st injection was a stable FCA emulsion in sodium chloride (50:50, v:v) for all the animals; 2nd injection was the test sample for treated animals and sodium chloride for control animals; 3rd injection was the test sample diluted in stable FCA emulsion and sodium chloride (25:25:50, v:v:v) for treated animals and sodium chloride diluted with stable FCA emulsion (50:50, v:v) for control animals. After 7 days from the experiments, test sample or sodium chloride (0.5 ml) was applied to the skin of the treated and control animals, respectively, for a period of 48 h (Michel Assad et al., 2002).

At day 21, the challenge phase was carried out by applying on the right side and the left side of the back of the treated and control animals, respectively, the test sample (0.5 ml) and sodium chloride (0.5 ml) for 24 h. At day 24 and 25, the reaction of both the treated and the control animals were evaluated.

2.8. Treatment of skin burns: in vivo studies

Studies were carried out on Sprague-Dawley, male rats aged 8–12 weeks and weighing 230–250 g (Charles River, Calco, Como, Italy). Animals were housed in an air-conditioned room at 22 ± 3 °C, 55 ± 5% humidity, 12 h light/dark cycles and allowed free access to water and food for the duration of the studies. All experiments were conducted in accordance with the guidelines of the European Communities Council Directive 86/609/EEC for care and use of experimental animals. 12 h before experiments rats were fasted but had free access to water during this time.

Rats were anaesthetized by intraperitoneal administration of equithesin (n = 6 per group), shaved on the back and burned putting a circular metallic plate (Ø 1.5 cm) heated at 80 °C, for 15 s, on the shaved back skin (day 1). The skin was then treated for 10 s with saline solution at 4 °C. Negative control rats were not burned (n = 6). The first group of burned rats was treated with sterile saline (positive control, n = 6), the second one with sterile saline and *R. ulmifolius* ointment (n = 6), and the third one with sterile saline and the commercial product Connettivina® (n = 6).

All tested samples (q.s.), were topical applied over the same dorsal site 2 times per day for 15 days. A picture of burned skin of each rat was taken (using a Coolpix, Nikon, USA) at 1, 3, 5, 7, 10, 13 e 15 day. Pictures were analyzed using an image software (Digimizer 4.2, MedCalc Software, Belgium) to measure the burn area and its reduction. The wound healing rate was expressed as percentage of wound contraction rate (WCR%) according to the equation $\text{WCR}\% = \frac{(A_0 - A_t)}{A_0} \times 100$, where $A_0$ was the burn area at time zero and $A_t$ was the burn area at time t.

At the end of the experiments, animals were sacrificed, complete blood was performed intracardially, while liver and kidneys were excised, fixed with paraformaldehyde (4%), and sectioned using a cryostat. Tissue specimens were stained with haematoxylin and eosin (H&E) and examined under the light microscope. Clinical biochemical analyses of the blood were carried out using an automatic biochemical analyzer (BS-120 Chemistry Analyzer, Mindray Medical International Limites, Milan, Italy), to measure parameters of liver and renal function. Measurement of prothrombin time and fibrinogen was performed using a coagulometer (ACL 7000, IL Instrumentation Laboratory, Milan, Italy).

2.9. Statistical analysis of data

Results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) and Scheffe’s test for multiple comparisons of means were performed using IBM SPSS statistics 20 for Windows.

3. Results and discussion

Traditional medicine has been used in Sardinia until the Second World War as a main health-care system. Nowadays, traditional remedies are still widely used and have given rise to a renewed interest in the scientific community. Different health problems may be treated following the folk medicine, but inflammatory diseases and burns of different degrees are among the most common health problems treated with traditional remedies. *R. ulmifolius* species have been traditionally used for different therapeutic purposes (Leonti et al., 2009) and have been especially applied onto burns of different degree due to their ability to promote the burn healing. Taking into account formulations of the traditional Sardinian medicine, the present investigation was aimed to design a new suitable, potentially industrial ointment based on *R. ulmifolius* shoot oleolite. Two different oleolites were obtained at different temperatures and qualitatively and quantitatively characterized. The one mostly rich in phytocomplex was used to prepare the industrial ointment, enriched with the olive oil unsaponifiable fraction, whose in vitro biocompatibility and in vivo ability to promote the burn healing were tested.

In Sardinian folk medicine, fresh *R. ulmifolius* shoots (25 g) are extracted in extra virgin olive oil (75 g) at ∼150 °C for 2 min and incorporated in lard. To obtain a potential commercial preparation, the dried shoots were used after confirmation that the drying process did not affect shoot composition. The loss on drying to constant mass was 75% and, therefore, the oleolites were prepared using 7.5 g of dried shoots in 92.5 g of olive oil. Two different oleolites were obtained: the first one by treating the dried shoots in hot olive oil (150 °C) and the second one by extracting the active ingredients in olive oil at room temperature (25 °C) for 10 days. Results demonstrated a superior ability of the extraction at room temperature than that at 150 °C, probably because at this high temperature many components were degraded. In fact, in the 150 °C oleolite
chromatogram (Fig. 1A) the most intense peaks were those corresponding to olive oil (61–64 min). On the contrary, polyphenolic peaks (52–57 min) were very small while they were clearly evident in the oleolite obtained at 25 °C (Fig. 1B). Taking into account these results, preparation method at 25 °C was selected to obtain the potential commercial product and further investigation was carried out to identify the most abundant components presented in 25 °C.

To better separate the polyphenolic components (Fig. 1B, left part of chromatogram), HPLC analysis was carried out using a gradient. Five different distinct and well-defined peaks were obtained (Fig. 1C). The components of the 5 fractions were separated and identified by HPLC–MS using standard references (Fig. 2 and Table 1). The most abundant components were kaempferol, an aglycone flavonoid and caffeic acid, a polyphenol whose antioxidant activity is well known.
Fig. 2. Mass spectrometry spectra of 5 main components of R. ulmifolius oleolite: caffeic acid (A), kaempferol (B), rhamnetin (C), quercetin (D) and isoquercetin (E).
According to the Sardinian folk medicine, the obtained oleo- lute was used to prepare suitable topical formulation (40 g/100 g of ointment) using anhydrous lanolin, white wax, olive oil, *Olea europaea* oil unsaponifiables, sterile water, butylated hydroxyanisole as antioxidant and sodium benzoate as a preservative. *Olea europaea* oil unsaponifiables are the insoluble fraction of extra virgin olive oil, especially rich in tocopherol and other phenolic compounds, whose antioxidant and protective effects are well known (Visoli et al., 2002; Waterman and Lockwood, 2007).

The ability of the formulations to scavenge DPPH free radical was assessed in vitro (Table 2). The antioxidant activity of both *R. ulmifolius* oleo- lute and ointment was similar to that of the quercetin methanolic solution used as reference, due to its well known scavenging ability (Villaño et al., 2007) and also because quercetin is one of the main components of *R. ulmifolius* oleo- lute (Villaño et al., 2007; Zheng and Wang, 2001). These results are encouraging because they confirm that the ointment formulation did not modify the polyphenolic antioxidant ability of the oleo- lute and that its properties are similar to those of pure quercetin at the same concentration.

Cell culture models are crucial to understand different mechanisms concerning cellular growth, differentiation, and numerous disease states. The use of a cell culture model has the advantage of providing a controlled environment to study a wide variety of cellular phenomena. For this reason, the cytotoxicity of the final ointment was tested using 3T3 fibroblasts in exponential phase of growth, by direct contact test (Carboni et al., 2013; Manca et al., 2013). Generally, direct contact methods have various benefits because they mimic physiological conditions, the zone of diffusion represents a concentration gradient of toxic chemicals and in addition, they require no extraction procedure. However, the major difficulty of this assay is the risk of physical trauma to cultured cells from either sample movement or crushing due to sample weight. Both qualitative and quantitative cytotoxicity evaluations were carried out. The qualitative assessment was performed observing cell culture by an inverted microscope, while the quantitative measurements were carried out using the Neutral Red Uptake method (NRU).

From the qualitative point of view, the positive control (latex) induced a severe reactivity, which extended more than 1 cm beyond the specimen, while no detectable zones around or under the *R. ulmifolius* sample could be found. Therefore it was confirmed that the ointment application did not cause any malformation or cell degeneration, Fig. 3. In addition, qualitative results were confirmed by quantitative analyses. Indeed, cells treated with the latex samples showed a very high mortality levels, which was around 96% after 72 h of experiment. On the contrary, the mortality of cells treated with *R. ulmifolius* ointment was very low reaching just 9% after 72 h experiment (Fig. 4) without any statistical differences at different times tested.

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>C/O (mg/g)</th>
<th>MW (g/mol)</th>
<th>MP (°C)</th>
<th>UV absorbance (nm)</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>0.5600</td>
<td>180.16</td>
<td>179</td>
<td>243–299</td>
<td>12.137</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.89400</td>
<td>286.24</td>
<td>285</td>
<td>263–367</td>
<td>26.075</td>
</tr>
<tr>
<td>Rhamnetin</td>
<td>0.0570</td>
<td>316.26</td>
<td>315</td>
<td>255–373</td>
<td>26.594</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.0680</td>
<td>302.24</td>
<td>301</td>
<td>255–366</td>
<td>27.540</td>
</tr>
<tr>
<td>Isoquercetin</td>
<td>0.0054</td>
<td>464.38</td>
<td>337–463</td>
<td>255–366</td>
<td>27.065</td>
</tr>
</tbody>
</table>

**Table 2**

In **in vitro** antioxidant activity (DPPH test) of quercetin dispersion, *R. ulmifolius* oleo- lute and *R. ulmifolius* ointment. Each single value represents the average ± standard deviation (SD) of at least six determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Antioxidant activity ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>QUE dispersion</td>
<td>51 ± 3</td>
</tr>
<tr>
<td><em>R. ulmifolius</em> oleo- lute</td>
<td>54 ± 5</td>
</tr>
<tr>
<td><em>R. ulmifolius</em> ointment</td>
<td>50 ± 4</td>
</tr>
</tbody>
</table>

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**Fig. 3.** In **in vitro** qualitative cytotoxicity of *R. ulmifolius* ointment (A) and positive control (latex film) (B) on 3T3 fibroblasts after 72 h treatment.

**Fig. 4.** In **in vitro** toxicity of *R. ulmifolius* ointment placed on filter paper and positive control (latex film) on 3T3 fibroblasts at 8, 24, 48 and 72 h, in comparison with negative control (100% viability). Each value represents the mean ± standard deviation (SD) of at least three determinations.

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To confirm the biocompatibility of the *R. ulmifolius* ointment, the hypersensitivity test was performed *in vivo* using guinea pigs. Both induction and challenge phases were evaluated after 7 and 21 days, respectively. Regarding the induction phase, after 7 days of induction, test sample or saline solution was applied locally for 48 h and, after this time, no abnormalities were observed in animals treated with *R. ulmifolius* ointment and even in the not treated ones. The same results were obtained performing the challenge phase test, in which the intradermal injection treatment was longer (21 days) and followed by local application of the samples for 24 h. Also in this case, no visible changes were detected in animals treated with *R. ulmifolius* ointment confirming the not sensitizing effect of this formulation.

The absence of toxic effects on fibroblast cell line and *in vivo* lack of hypersensitivity phenomena, encouraged the furtherance of the study to evaluate the *in vivo* efficacy in animals of the *R. ulmifolius* ointment following topical administration. In particular, the ability of the *R. ulmifolius* ointment to promote tissue regeneration in second-degree burn was investigated. A commercial formulation Connettivina® (hyaluronic acid) and/or saline solution were used as controls.

Burned Sprague-Dawley rats were subjected to chronic topical treatment for 15 days. No outward signs of pain or discomfort were noted in the animals and their behaviour (e.g., food intake, daily activity) appeared normal. All the tested animals showed formation of a crust on skin wound that lasted for several days, leaving a residual lesion on the skin tissue after its fall (Fig. 6). Compared to the other treatments, *R. ulmifolius* ointment showed a quick reduction of wound depth during the first 5 days post-injury and a faster reduction in wound area, reaching the almost complete reepithelization at days 13 and 15. Data were confirmed by wound contraction ratio (WCR%) measurements (Fig. 5), after 13 and 15 days WCR% of animals treated with the *R. ulmifolius* ointment was statistically higher than that of animal treated with saline solution or Connettivina® (*P* < 0.01 at 13 days and *P* < 0.05 at 15 days). Clearly, *R. ulmifolius* ointment showed a superior ability in skin restoring due to the synergic activity of its heterogeneous components (polyphenols of *R. ulmifolius*, unsaponifiables of olive oil, and lanolin lipids), which contributed together to favour restoration of the damaged tissue and lesion reepithelization. In particular, bearing in mind previous findings on oleolite reepithelization, we can suppose that the good formulation activity was due to the presence of the different phenolic compounds such as polyphenols and flavonoids (Castangia et al., 2014; Caddeo et al., 2014). The latter have been widely used for topical treatments as anti-inflammatory and antioxidant agents in wound/burn healing. In particular, flavonoids and polyphenols are known to reduce the excess of protease and reactive oxygen species, often formed in

![Fig. 6. Digital pictures of representative burn wounds in Sprague-Dawley rats treated with *R. ulmifolius* ointment, saline solution and Connettivina® up to 15 days.](image_url)
the injured site, and to protect cells and extracellular matrix from oxidative damage by preventing or slowing down the cell necrosis progress and its potential neoplastic transformation (Surh, 2003; Amin et al., 2009). In addition, natural flavonoids and polyphenols are able to favour endothelial cell and fibroblast proliferation and migration. Therefore, they are capable of leading the formation of new blood vessels and capillaries, regenerating new dermal tissue and remodelling the newly formed tissue by stimulating the collagen fibre production (Paolino et al., 2012; Sünart et al., 2010). Furthermore, these antioxidant molecules are able to endorse the wound-healing process thanks to their antimicrobial and astringent properties, which appear to be responsible for wound contraction and high rate of epithelization (Turkoglu et al., 2007; Sanwal and Chaudhary, 2011; Khaled-Khodja et al., 2014).

After chronic treatment, a general assessment of the health state of the animals was carried out measuring liver and kidney functionality. Particularly, parameters of liver, renal function and coagulation factors were measured at the end of the treatment. No significant modification on parameter values were detected with both R. ulmifolius ointment and saline solution. Likewise, microscopic changes were not highlighted in the structure of the liver or kidneys of the animals chronically treated with the R. ulmifolius ointment (data not shown).

4. Conclusion

During this work, starting from Sardinian folk medicine, in order to obtain a suitable commercial product from R. ulmifolius shoots, an innovative and optimized extraction method was developed and a polyphenol enriched oleolect was prepared. The oleolect extract was then incorporated in an ointment based on anhydrous lanolin, white wax and olive oil. Toxicity studies revealed the complete biocompatibility of the R. ulmifolius ointment and the absence of hypersensitivity after acute and chronic treatment of the tested samples. In vivo studies confirmed the effective activity of the R. ulmifolius ointment to restore burned tissue and facilitate skin regeneration. Thanks to these favourable properties, R. ulmifolius ointment may be clinically suggested for treating various types of burns/wounds.

References
