Tunisian *Clematis flammula* Essential Oil Enhances Wound Healing: GC-MS Analysis, Biochemical and Histological Assessment

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Abstract: The aerial part of *Clematis flammula* (*Ranunculaceae*) has been traditionally used in the treatment of skin diseases including mycotic infection in the Tunisian traditional medicine. The study was undertaken to extract and determine the essential oil chemical composition of *Clematis flammula* aerial parts and to assess the potential of anemonin in wound healing on mechanically wounded wistar rats. The essential oil was obtained by hydrodistillation and analyzed by GC-MS. Anemonin was isolated and then incorporated as active in a cream for which the cytotoxicity was evaluated by methyl thiazolyl tetrazolium (MTT)-based colorimetric assay. Then, its potential in wound healing on mechanically wounded wistar rats was assessed. The GC-MS analysis showed that the major compound was protoanemonin (86.74%) which spontaneously dimerised in part to form the anemonin. The wound healing activity of anemonin cream exhibited a non toxic potential of anemonin at a concentration of 25 µg/mL with a cell migration efficiency that reaches more than 80% after 48 hours of treatment. Wound healing efficiency was evaluated by monitoring morphological and skin histological analyses. Comparable wound surface reduction of the group treated by anemonin cream (\(p \geq 0.05\)) when compared to the reference treated group. The skin histological analysis showed the completely wound closure. Antioxidant activity was assessed by the malondialdehyde (MDA) rates and antioxidant enzymes (glutathione peroxidase (GPx) and catalase) determination. The results provided strong support for the effective wound healing activity of anemonin cream, making it a promising candidate as a therapeutic agent in tissue repairing processes.

Key words: *Clematis flammula*, essential oil, anemonin, wound healing, antioxidant, Cytol Centella

1 Introduction

Aromatherapy, which uses essential oils extracted from different plants and herbs, is widely employed and is becoming a major alternative in complementary medicine. Last years, inhalation aromatherapy has particularly got attention especially for its effects of emotional or psychological conditions improvement. Various clinical assessments have proved the aroma-therapy potential for worry\(^1\), insomnia\(^2\), exertion\(^3\) and pain\(^4\). Regarding anxiety, efficiency on the clinical results is partially supported by few studies demonstrating the essential oils beneficial effect\(^5,6\) and a metabolic response to essential oils inhalation in anxiety model rats\(^7\). Furthermore, the purposes of aromatherapy and its therapeutic potentials have been developed from emotional and psychological symptoms to various physical diseases, some essential oils were proved to act as anti-inflammatory\(^8\), anti-viral\(^9\), anti-tumor\(^10\), antihyperglycemic\(^11\) and anti-carcinogenic\(^12\) agents. Wound healing is one of the expected targets of essential oils topical application\(^13\)–\(^17\).

The species *Clematis flammula* is widely distributed in the temperate regions of Europe and Asia as well as in the high tropics\(^18\) in China, Australia, New Zealand, India,
North America, South America, North Africa, the Himalayas and Japan\textsuperscript{19}. In folk medicine, the aerial parts of different \textit{Clematis} species are particularly used to treat blisters, and as a poultice for festering wounds and ulcers\textsuperscript{20}.

Indeed, this study aimed to identify the chemical composition of essential oil extracted from Tunisian \textit{C. flammula} and to determine whether application of anemonin to the wound bed reduces healing time and improves local pain associated with this pathology; any adverse effects or reactions related to its application were also investigated.

### 2 Experimental

#### 2.1 Plant Material

\textit{Clematis flammula}, in full bloom, was collected in June 2014 in the Kasserine province (Tunisia). Voucher specimens (LCSN 119) were authenticated by Pr. Mohamed Chaieb and have been deposited in the Laboratory of Organic Chemistry LR17ES08 “Natural Substances Team” of the Faculty of Sciences of Sfax (Tunisia).

The reference drug (Cytol Centella, Cytolnat laboratory) was purchased from a local pharmacy at Sfax (Tunisia). It included water, hydrogenated polydecene, glyceryl stearate, propylene glycol, \textit{Vitis vinifera} seed oil, palmitic acid, stearic acid, squalane, hydrogenated vegetable oil, \textit{Centella asiatica} leaf extract, \textit{Persea gratissima} oil, algin, glycine, soybean oil, triethanolamine, \textit{Arachis hypogaea} oil, cetyl palmitate, allantoin, ethylhexyglycerin, tocopheryl acetate, caprylyl glycol, retinyl palmitate, disodium EDTA, tocopherol, phenethyl alcohol and perfume.

#### 2.2 Essential oil and cream preparation

The \textit{C. flammula} aerial part was collected and ground, then submerged in water in a 1 L round bottom flask and finally submitted to hydro-distillation in a glass Cleveger type bottle allowing transparent light yellow oil separation. The extraction was carried out for 3 hours. After cooling, the water and essential oils were separated. The oily fraction was decanted to be used as essential oil. To improve the essential oil’s recovery, extraction of the aqueous phase was achieved with ethyl ether (Merck)\textsuperscript{21}. Then, the organic phase was dried over anhydrous sodium sulphate (Merck). The recuperated essential oil was stored in a dark glass bottle at 4°C prior to GC and GC–MS analyses. The essential oils extraction yield of the \textit{C. flammula} was 1.41%.

After incubation of this essential oil for few hours, we got white crystals that were formed at the bottom of the tube. The crystals were subjected to column chromatography in order to isolate the anemonin compound. Then, a cream based on it (0.012%) was prepared as described in the formulation on Table 1 in order to determine the healing effect of this molecule.

<table>
<thead>
<tr>
<th>Active compound</th>
<th>Anemonin (0.012 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase</strong></td>
<td><strong>Components</strong></td>
</tr>
<tr>
<td>A : Aqueous</td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td>Glycerin</td>
</tr>
<tr>
<td></td>
<td>Propylene glycol</td>
</tr>
<tr>
<td></td>
<td>Sodium benzoate</td>
</tr>
<tr>
<td></td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td></td>
<td>C13-14 Isoparaffin</td>
</tr>
<tr>
<td></td>
<td>Laureth-7</td>
</tr>
<tr>
<td>B: Oily</td>
<td>Sunflower oil</td>
</tr>
<tr>
<td></td>
<td>Sesame oil</td>
</tr>
<tr>
<td></td>
<td>Isopropyl myristate</td>
</tr>
</tbody>
</table>

#### 2.3 Instrumentation and analyses

The essential oil analyses were performed using GC-MS analyzer. An Agilent 19091S-433 HP-5MS 5% phenyl methyl siloxane capillary column (30 m length, 0.25 mm diameter, 0.25 μm film thickness) was used. A 1 μL aliquot of the essential oil was injected into the column using a 10:1 split injection, at a temperature set up at 325°C. The GC program was initiated by a column temperature set at 40°C for 1 min, increased to 325°C at a rate of 4°C/min, held for 10 min. The helium was used as the carrier gas (0.8 mL/min). The mass spectrometer was used in the 70 eV EI mode with scanning from 41 to 450 amu at 0.5 s, and mass source was set at 200°C. The essential oil compounds were identified corresponding to mass spectral fragmentation patterns stored in the spectrometer database using the National Institute of Standards and Technology Mass Spectral database (NIST-MS, 1998)\textsuperscript{21}.

#### 2.4 Animals

A total of 15 female two month-old albino rats weighing between 170-185 g were purchased from the central pharmacy (Siphat, Tunisia). The animals were settled in plastic cages with stainless-steel grid taps. They were maintained under environmentally controlled conditions (26°C temperature; 60 % humidity and 12 hours light/dark cycle) with \textit{ad libitum} access to standard food (SICO, Sfax, Tunisia) and drinking water. The animals were handled in accordance with the present national guidelines on animal care and use.

The injured rats were divided into three groups of 5 animals each, as follows:

- **Group I**: untreated rats;
- **Group II**: rats treated with anemonin cream (0.012 %);
- **Group III**: rats treated with Cytol Centella as the reference drug;
- **Group IV**: rats treated with vehicle (cream non-containing anemonin).
2.5 Evaluation of cytotoxicity on keratinocytes

Primary keratinocytes cells (4 × 10⁶ cells/well) isolated from human skin were seeded into 24-well plates. After serum starvation for 24 h, the cells were incubated with test samples for 24 h at 37°C under 5% CO₂. The MTT (Sigma-Aldrich) solution (100 μL of 5 mg/mL) was added, and the plates were incubated for an additional 4 hours. The supernatant was eliminated, and the formazan crystals were dissolved in dimethyl sulfoxide (1 mL, Sigma-Aldrich). Finally, the optical density was determined at 540 nm using an ELISA reader (TECAN)²⁰.

2.6 Wound healing models

The C. flammula essential oil was tested for its wound healing activity in rats using an excision wound model. After being anaesthetized with an intra peritoneal injection of chloral hydrate (400 mg/kg), an impression was made by a round seal of 25 mm diameter on the dorsal thoracic region of the rats, 50 mm away from the ear and 10 mm away from vertebral column. The skin of impressed area was superficially excised to the full thickness to get a wound area of about 500 mm². Haemostasis was obtained by blotting the wound with cotton swab drenched in normal saline solution. 500 mg of each cream was gently applied onto the wounds with swab until recovering them by a thin layer. Contractions, which involve the wound closure in the first 2 weeks, were controlled by a transparent tracing paper. Wound area was checked by retracing the wound on a millimeter scale graph paper and then weighing the paper to estimate the areas. We used the following formula²¹ to calculate the degree of wound healing:

\[
\text{Contraction} (%) = \left[1 - \left(\frac{\text{wound area on corresponding day}}{\text{wound area on zero day}}\right)\right] \times 100
\]

The required number of days for complete contraction was registered; the epithelialization progress and the infection evolution were evaluated using macroscopic images.

2.6.1 Biochemical analyses

The rats from each experimental group were decapitated on the 14th postoperative day for determining the healing course in terms of biochemical characteristics. In addition, the granulation tissue part of all the different experimented rats including wound area, was removed in order to measure the tissue malondialdehyde (MDA) contents as well as the glutathione peroxidase (GPx) and the catalase (CAT) enzymes activities.

2.6.1.1 Lipid peroxidation measurement

The end product of polyunsaturated fatty acid peroxidation, the MDA, reacting with thiobarbituric acid (TBA) was determined according to Esterbauer²¹. Briefly, an aliquot of granulated tissue extract supernatant was mixed with 1 mL of 5% trichloroacetic acid and centrifuged at 2500 × g for 10 min. Then, we added one mL of thiobarbituric acid reagent (0.67%) to 500 mL of supernatant to be heated at 90°C for 15 min. The mixture was then cooled and its absorbance was measured at 532 nm using a spectrophotometer (Jenway UV-6305, Essex, England). The MDA rates were calculated using the 1,1,3,3-tetraethoxypropane as a standard and expressed as nmoles of MDA/mg of protein.

2.6.1.2 Determination of granulated tissue antioxidant enzyme activities

The GPx activity was given according to the commercial kit (Catalog no. RS 505; Randox, Ltd. Crumlin, United Kingdom). GPx catalyzes the gamma-glutamylcysteineyl glycine (GSH) oxidation by cumene hydroperoxide. In the presence of GSH reductase and NADPH, the oxidized GSH changed immediately to the reduced form associated with NADPH oxidation to NADP⁺. The decrease in absorbance at 340 nm was measured and the enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein. A total of 2 mL skin homogenate (about 1.5 mg proteins) was added to 1 mL phosphate buffer (0.1 M, pH = 7) containing 100 mM H₂O₂²³. The H₂O₂ rate was assessed by measuring the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated using an extinction coefficient of 0.043 mM⁻¹ cm⁻¹ and expressed in international units (I.U.), i.e., in μmoles of H₂O₂ destroyed/min/mg protein at 25°C. In all samples, the protein concentrations were measured according to the method of Lowry et al.²⁰ using bovine serum albumin as a standard.

2.6.2 Histological studies

The wounded skin part of the rats was maintained in formalin at 10% for histopathological examinations and processed in a series of graded ethanol solutions. They were then embedded in paraffin, serially sectioned at 5 μm and stained with hematoxylin–eosin²⁵.

2.7 Evaluation of anemonin cream irritant potential

This evaluation was conducted following the test "Pre-skink" developed by the company Eurosaf based on 2000/33/EC (25th April, 2000)²⁸.

2.8 Statistical analysis

The statistical analysis was realized using one-way analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD) test as a post-hoc test for comparison between groups. The results were expressed as mean ± standard deviation. Differences between means were considered significant at p values of less than 0.05.

3 Results

3.1 The essential oil Chemical composition

The chemical composition of the essential oil, extracted from C. flammula aerial part and identified by GC-MS, is
presented in Fig. 1 and codes are described in Table 2. The major compound in this essential oil was the protoanemonin (86.74%) which has also been detected in *C. jubata* and *C. montana*29,30.

3.2 Isolation of anemonin from *C. flammula* essential oil

After few hours of the essential oil storage at 4°C, white crystals precipitated which could be the result of the protaanemonin spontaneous dimerisation into anemonin and other artifacts such as ranunculin formation.

The NMR analysis in deuterated chloroform (CDCl₃) of crystals (E₁) showed the anemonin compound (Figs. 2 and 3) with other artifacts which were minors. For this reason, we tried to solubilize the crystals in a minimum of chloroform; we got the filtrate to be subjected to column chromatography (SiO₂, CHCl₃) later. The remaining crystals were dissolved in deuterated dimethyl sulfoxide DMSO-d₆ (Figs. 4 and 5); the NMR spectra showed a pure anemonin (E₂). This latter was identified by directly comparing its physical and spectral data (¹H NMR and ¹³C NMR) with the previously reported data32.

3.3 Evaluation of cytotoxicity on keratinocytes

The cytotoxicity was analyzed using an MTT assay. Cultured normal keratinocytes were incubated with increasing anemonin concentrations from 25 to 100 μg/mL (Fig. 6). The results showed that this compound became toxic at concentrations exceeding 25 μg/mL. Interestingly, lower
anemonin concentration (25 μg/mL) did not diminish the keratinocytes proliferation in comparison with the untreated cells (0 μg/mL; Fig. 6). A positive linear correlation was established between keratinocytes viability and anemonin concentrations (Viability (%) = [Anemonin], R² = 0.983).

3.4 Wound healing activity
The wounds photographic representations of all experimented groups are shown in Fig. 7. From the fourth day of healing, it was noted the presence of a large inflammatory bulb in the control group with whitish punctuation, while the wound surface observed for anemonin and Cytol Centella treated rats were smaller.

On the ninth day of healing process, some crusts started falling down revealing a red granulated tissue with distant sides in the control (no treated wound) and pinkish for both treated groups with anemonin and Cytol Centella creams. In addition, the strands became narrow because of a more advanced re-epithelialization. The continuous proliferation of the edges epithelial cells was exhibited at the thirteenth day to the complete wounds closure in the group treated by Cytol Centella. However, the closure was almost com-

\[ \text{Table 2} \quad \text{Mean percentages of the fresh aerial part essential oil components from } \text{Clematis flammula (Kasserine, Tunisia)} \text{and their Kovats indexes.} \]

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Compound</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>KI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Structure</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protoanemonin</td>
<td>7.86</td>
<td>575</td>
<td><img src="image1" alt="Structure" /></td>
<td>86.74</td>
</tr>
<tr>
<td>2</td>
<td>Gamma-Valerolactone</td>
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<tr>
<td>3</td>
<td>3-Hexen-1-ol</td>
<td>11.22</td>
<td>611</td>
<td><img src="image3" alt="Structure" /></td>
<td>0.19</td>
</tr>
<tr>
<td>4</td>
<td>1-Octanol</td>
<td>13.56</td>
<td>863</td>
<td><img src="image4" alt="Structure" /></td>
<td>0.23</td>
</tr>
<tr>
<td>5</td>
<td>Linalool L</td>
<td>14.40</td>
<td>1082</td>
<td><img src="image5" alt="Structure" /></td>
<td>0.97</td>
</tr>
<tr>
<td>6</td>
<td>Borneol</td>
<td>16.55</td>
<td>1179</td>
<td><img src="image6" alt="Structure" /></td>
<td>0.74</td>
</tr>
<tr>
<td>7</td>
<td>p-menth-1-en-8-ol</td>
<td>17.40</td>
<td>1218</td>
<td><img src="image7" alt="Structure" /></td>
<td>0.85</td>
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<tr>
<td>8</td>
<td>Lavandulyl acetate</td>
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<td>1235</td>
<td><img src="image8" alt="Structure" /></td>
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<tr>
<td>9</td>
<td>Thymol</td>
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<td>1237</td>
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<tr>
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<td>3-methyl-4-isopropylphenol</td>
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<td>1260</td>
<td><img src="image10" alt="Structure" /></td>
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<td>Phenol</td>
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<td>1222</td>
<td><img src="image11" alt="Structure" /></td>
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<tr>
<td>12</td>
<td>3-methyl-1-phenyloxindole</td>
<td>35.83</td>
<td>1619</td>
<td><img src="image12" alt="Structure" /></td>
<td>1.32</td>
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<tr>
<td>13</td>
<td>7,9-di-torr-buty-1 oxaspiro (4,5) Deca - 6,9-diene - 2,8-dione</td>
<td>37.21</td>
<td>1765</td>
<td><img src="image13" alt="Structure" /></td>
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<tr>
<td>14</td>
<td>Mono-(2-ethyl-hexyl) ester</td>
<td>51.82</td>
<td>2150</td>
<td><img src="image14" alt="Structure" /></td>
<td>1.43</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>98.80</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup>Kovats Index: calculated against C5-C18 n-alkanes.
Fig. 2 $^1$H-NMR spectrum of unpurified crystals (CDCl$_3$, 400 MHz).

Fig. 3 $^{13}$C-NMR spectrum of unpurified crystals (CDCl$_3$, 100 MHz).
Fig. 4 $^1$H-NMR spectrum of purified anemonin (DMSO-$d_6$, 400 MHz).

Fig. 5 $^{13}$C-NMR spectrum of purified anemonin (DMSO-$d_6$, 100 MHz).
Figure 8 shows the wound area evolution recorded for the control, the anemonin and reference cream treated groups during the healing process. The cream based on anemonin (0.012%) displayed a marked wound-healing potential. In fact, the anemonin and the reference cream showed comparable effects throughout the healing period ($p \geq 0.05$). However, no comparable wound area evolution was observed between the four groups except at the first day.

Anemonin cream would be responsible for the significant wound area decrease that was comparable to that of Cytol Centella. However, the surface variation displayed by the anemonin and Cytol Centella treated groups followed a polynomial function. Therefore, it could be concluded that the anemonin and Cytol Centella creams induced kinetics changes in the rats wound healing.

3.4.1 Histopathological study

On the thirteenth day of the treatment, the tissue section microscopy of the skin showed epidermal and dermal regenerations in groups II and III (Fig. 9). The skin autopsy of the untreated rats exhibited uncompleted re-

<table>
<thead>
<tr>
<th>Healing duration</th>
<th>Untreated group</th>
<th>Anemonin treated group</th>
<th>Cytol Centella (Reference group)</th>
<th>Vehicle treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
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<tr>
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<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
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</tr>
<tr>
<td>D7</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
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<tr>
<td>D9</td>
<td><img src="image13" alt="Image" /></td>
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<tr>
<td>D11</td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
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<tr>
<td>D13</td>
<td><img src="image21" alt="Image" /></td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
</tr>
</tbody>
</table>

Fig. 6  Anemonin concentration effect on keratinocyte cytotoxicity.

*: Significant difference in comparison with control group (0 μg/mL).

Fig. 7  The wounds photographs of different groups during the healing process.
Clematis Flammula Essential Oil Enhances Wound Healing in Wistar Rats


The topical anemonin cream application displayed remarkable antioxidant enzyme activities and the lipid peroxidation in comparison with the untreated group at different days. The anemonin and reference creams treated groups were comparable to that of the normal skin. The histological observations of treated skins showed a more advanced epithelial regeneration, well-organized layers of dermis and epidermis and faster keratinization with complete fibrous connective tissue regeneration in the dermis while compared to the untreated group.

3.4.2 Wet granulation tissue antioxidants and MDA

The anemonin cream topical application effects on the antioxidant enzymes and the lipid peroxidation are shown in Figs. 10 and 11. The antioxidant enzymes levels were determined because it has revealed that they enhance wound healing process.

Accordingly, wound induction led to a significant decrease in the antioxidant enzymes activities accompanied with a significant increase in the MDA rate. However, topical anemonin cream application displayed remarkable antioxidant enzyme activities which were similar to those displayed by the reference animals. Compared to the untreated rats, the anemonin treated group showed significant increases in CAT and GPx activities, respectively of 60% and 100%. In contrast to the antioxidant enzyme levels, the MDA rates increased in the injured rats (groups II). Indeed, wound displayed an MDA rate increase of 60.22% while compared to that of the normal group (group I). However, the anemonin cream treatment reestablished the MDA level to its normal value (group I).

3.4.3 Irritant potential of anemonin cream assessment

In vitro, the cream application on a fresh skin didn’t cause any significant cell death. Indeed, the viability was of 80% relative to control. As a result, the cream including anemonin at 0.012% was non-irritating for the experimented rat’s skin.

4 Discussion

The composition of the essential oil may be affected by many factors, especially the vegetal developmental stage, the extraction method used and the chemical composition analysis conditions. In comparison to other Clematis, we found that the Tunisian C. flammula essential oil (Fig. 1) is very rich in protoanemonin (86.74%), a compound previously found in C. hirsuta, C. sinensis and C. wightiana (Saudi Arabia) with respectively yields of 26.56%, 4.57% and 28.66%, much lower than that found in the present study. The steam distillation gave protoanemonin yields of 0.12, 0.05 and 0.67% from fresh whole plant of the Australian Clematis of the species microphylla, glycynoides and aristata, respectively. Therefore, the genus Clematis revealed the presence of quantitative variation in the protoanemonin yields. Moreover, a difference was noted in the essential oil constituents with a specific species major component; while the 5-hydroxy-methyl-2-furaldehyde and the vanillic acid were found to be the main compounds in the essential oil of C. armandii, they were absent in the Australian Clematis species. In the present study, the other constituents of the essential oil are minor (Table 2). Among these compounds, the 3-methyl-1-phenyloxindole is an oxindole characterized by an extensive range of biological effects, including antimicrobial, antiproliferative, anticancer, anti-inflammatory and antihypertensive activities. Indeed, the oxindole derivatives are responsible of various biological activities which include the antiviral and antifungal potentials. The 7,9-di-tert-butyl-oxaspiro (4,5) dec-a-6,9-dien-2,8-dione, a ketone known for its antimicrobial effect, was also found; it is considered as an important antimicrobial agent.

During the essential oil storage, the spontaneous dimerization of protoanemonin gave the anemonin as a main compound. The NMR analysis of anemonin in CDCl₃
Fig. 9 Microscopic view (Hemotoxylin & Eosin stain) of healing wound tissue and epidermal/dermal re-modeling in different animal groups at day 13.
The microscopic observation is made at a magnification of x40, x100 and x400. A: epidermis, B: Dermis, C: hypodermis. U: ulcer; Re: re-epithelization, K: keratinization.
Fig. 9  Continued.
Fig. 10  Variation of GPX and CAT activities in skin tissue, liver and erythrocytes (control and treated rats).
G0: control rats without wounds, G1: rats with untreated wounds, G2: rats with wounds treated with anemonin cream, G3: rats with wounds treated with Cytol Centella. a: \((p \leq 0.05)\) : significant variation compared to control rats without wounds (G0), b: \((p \leq 0.05)\) : significant variation compared to wound rats (G1).
2 and 3) and in DMSO-d$_6$ (Figs. 4 and 5) showed its better solubility in dimethyl sulfoxide.

Considering the medicinal application of preparations to treat skin lesions, particularly burns and wounds, several plants have been experimentally studied on various animal models and were reported to display promising wound healing activity$^{42,43}$.

Regarding this chemical characteristic, the wound healing evaluation of anemonin showed that it could easily penetrate the skin layers. Indeed, it was recently demonstrated that the tested permeability on an in vitro human skin model of this small molecule (192 g/mol) was of 41.6% which enhanced the component penetration in order to reach the different skin layers$^{44}$. Taking into account this absorption rate, we can estimate the quantity absorbed which was equal to 25 μg per wound surface (4 cm$^2$) in the present study. This confirms the efficiency of the anemonin formulated cream (Table 1).

In the wound healing evaluation, the noted contraction rate of the anemonin cream (0.012%) treated rats was significantly higher than that of the control group as shown in Fig. 8. This result provides further support for the effectiveness of anemonin cream in wound healing.

Moreover, the wounds treated with anemonin cream showed less whitish punctuations than those of the other groups (Fig. 7). This result could be explained by the cream antimicrobial potential, due to the anti bacterial and antiviral activities of the anemonin previously evidenced$^{45}$.

Furthermore, during all the healing process, the anemonin cream exhibited notable activity comparable to the reference drug ($p > 0.05$). This wound healing potential is attributed only to the anemonin. Indeed, the vehicle cream...
(without anemonin) did not show significant healing activity when applied in the same conditions (Figs. 7 and 8). Moreover, the reference healing activity was not only attributed to Centella asiatica leaf extract, but to other several active ingredients, among which two vegetable oils (Persea gratissima oil and Vitis vinifera seed oil) that are well known for their skin regeneration effects. It also contains allantoin, which is known as cell proliferation-stimulating agent and squalene used in the treatment of skin disorders. In addition, the skin wound excision autopsies (Fig. 9) observed for the treated groups at day 13 showed healed skin structures with more advanced epithelial regeneration compared to the control group. Well-organized dermal and epidermal layers were observed, whereas the untreated group lagged behind the treated group in terms of different layer formations. The epidermal thickening observed for the latter group could presumably be due to the hyper-proliferative state of keratinocytes and their growth. Comparatively, the anemonin cream and the reference treated groups showed almost the same epidermal thickness when considering that of healthy skin. This reflected the more advanced keratinocytes cell proliferation of the treated group. The wound area reduction could be attributed to the anemonin biological activities, especially the antioxidant and the anti-inflammatory activities. Furthermore, transforming growth factor-beta (TGF-beta) is well known as a key signal in orchestrating wound repair. The canonical TGF-β signaling pathway is mediated by the Smad family of proteins. When TGF-β reaches the membrane of target cells, it binds directly to TGF-β type II receptors (TbRII), which leads to the recruitment of TGF-β type I receptors (TbRI). Then, the TbRII trans-phosphorylates TbRI enabling the TbRI kinase domain to act on cytoplasmic proteins and thereby propel Smad proteins dependent signaling pathways. Recently, it was demonstrated that, in an LPS-induced intestinal injury pig model, the anemonin supplementation has increased the gene expressions of TbRII, Smad4 and Smad7 after the LPS challenge. This may indicate that the canonical Smads signaling pathway was activated. Therefore, it could be hypothesized that the beneficial role of anemonin in wound healing activity may be partially influenced by the TGF-β 1.

Further results revealed that the anemonin cream topical application increased proteins content, indicating active protein matrix synthesis and its deposition throughout the granulation tissues as well as in the cell hyperplasia. This would demonstrate the scar width reduction by cell proliferation and collagen formation, while Rasik and Shukla mentioned ROS overproduction and hence, lipid peroxidation. The increase in the thiobarbituric acid reactive substances (TBARS) rate reflects the excessive production of free radicals, which leads to an increase in the markers of oxidative stress. It therefore seems that the wound induction stimulates the main sources of free radicals which are the mitochondrial respiratory chain and NADPH oxidase causing the formation of the superoxide radical.

Based on present results, we noted a close relationship between edema size change and lipid peroxidation with a correlation coefficient of less than 1. Indeed, the TBARS level increase in injured rats inflammation is related to the maintenance of edema size while its decrease is related to the decrease of the TBARS rate (Fig. 11). This relationship explains that the lipoperoxidation hinders the anti-inflammatory activity. Therefore, it would be interesting to explore the effect of anemonin cream on antioxidant status.

The pathogenesis of several diseases such as inflammation is associated with an overproduction of reactive oxygen species (ROS) which generates an imbalance of the oxidative stress. The cells respond by developing a defense mechanism based on the production of antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPx). In order to explore the status of the oxidative balance, the activities of these two antioxidant enzymes on liver, erythrocyte and tissue and levels were evaluated (Fig. 10).

These results suggest that topical application of anemonin cream participates in cellular protection not only in a direct way as a source of antioxidant molecules but also indirectly as an activity stimulator and antioxidant enzymes exprssor. These results show that there is a relationship between the variation of TBARS and antioxidant enzymes. Indeed, the increase of the antioxidant enzymes activity through the topical application of anemonin cream is coupled with the decrease of TBARS rate and inflammatory edema size. Therefore, the wound healing activity could be due to the anemonin antioxidant activity while it acts as inhibitors of cyclooxygenase and as scavengers of free radicals generated during the healing process.

5 Conclusion

In the present work, the healing potential of anemonin isolated from Clematis flammula essential oil has been investigated on rat model, considered as a convenient method to assess medicinal derivatives wound healing efficiency; its experimental investigation may permit the findings transposition to human beings. The topical application of anemonin cream stimulated wound contraction, increased antioxidant enzymes activities and regenerated skin tissues in the treated as compared to the control group of rats. These results suggest an efficient healing power of anemonin cream that could be attributed to its anti-inflammatory property.

It would be interesting to develop other complementary research, dealing with the optimization of the anemonin
concentration to be used for skin healing in order to establish the cream formulation. Furthermore, the investigation of other forms (lotion, gel, ointment...) would be interesting to consider in designing an effective and marketable product.

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Author Contributions

Rakia Saidi, Ferdaws Ghrab, Tahya Boudawara, performed the experiments. Kim Kallel, Emna Ammar, Abdel-fattah El Feki, Raoudha Mezghani Jarraya, Christophe Chesné designed respectively the chemical experiments and the biological experiments. All the authors analyzed the data. Rakia Saidi, Ferdaws Ghrab, Emna Ammar and Raoudha Mezghani Jarraya wrote the paper.

All authors reviewed and approved the final manuscript.

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