An Innovative Ointment Made of Natural Ingredients 
with Increased Wound Healing Activity

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Abstract

The authors present and evaluate an original product in the armamentarium of the preparations for burns and wounds treatment, made solely on the basis of medicinal plants and natural ingredients. The ointment formulation comprises olive oil extract from a mixture of nine medicinal plants with wound healing activity (Calendula officinalis L., Matricaria chamomilla L., Symphytum officinale L., Hypericum perforatum L., Achillea millefolium L., Arctium lappa L., Plantago major L., Althaea officinalis L., Quercus robur L.), sea buckthorn oil, lavender essential oil and as thickening agents, coconut oil, beeswax and conifer resin. The LC-MS analyses of the ethanolic extracts from the plant mixture and of the ethanolic re-extracts from oil plant mixture extract and from ointment have evidenced high levels of polyphenols like caffeic, chlorogenic, gallic and ferulic acids, as well as quercetin and rutin, all of which being known compounds with good wound healing activity. The neutral red assay has shown no cytotoxic effect on fibroblast NCTC cell line exposed to herbal extracts. Finally, the wound healing action of the submitted ointment has been clinically confirmed and highlighted by some case reports.

Keywords: wound healing, antioxidant activity, plants extracts, LC/MS, fibroblasts

1. Introduction

Wound healing involves a complex, dynamic process, leading to the restoration of the skin anatomical continuity and function (1) and engages interactions of extracellular matrix mediators and different cells like fibroblasts and keratinocytes. In the healing process reactive oxygen species (ROS) are also generated and their presence may cause cellular damage by different mechanisms such as peroxidation of membrane lipids (2, 3). This suggests that the presence of the antioxidants in wounds and burns treatment may be favorable for healing (3).

Currently, a lot of research and studies are performed worldwide to discover a range of new products and formulations, as well as the best methods of wounds treatment. The pharmaceutical products commonly used today in wound care armamentarium are chemically synthesized and may have different side effects. For example, the silver sulfadiazine (SSD) cream, topical drug of choice and gold standard in burns treatment, delays the wound healing, slows down the epithelization rate, in prolonged conservative treatment leads to healing with hypertrophic or atrophic scars, may have local cytotoxic activity (on keratinocytes and fibroblasts) and may cause systemic complications including neutropenia, erythema multiform, renal toxicity and methemoglobinemia (4, 5).
An alternative to such synthetic products is offered by the medicinal plants and the natural remedies, which have been used with confidence by mankind from ancient times to treat various skin diseases including burns or wounds. Medicinal plants application for wound healing is actually supported by many scientific studies (6-13) and has many advantages: is cheap, affordable, effective, easy to manage, also safe as side effects are rare and minor, mainly local hypersensitivity. The plants used in traditional medicine contribute to wound healing and tissue regeneration by multiple mechanisms, which still need assessment and validation by scientific studies. The medicinal plants are a tremendous resource for the management and treatment of the wounds, by the bioactive compounds from the plant material, of which polyphenols are representatives. For example, gallic acid has antimicrobial, antifungal and antioxidant activity (14, 15). Caffeic acid and their esters derivatives are mentioned as anti-inflammatory agents (2), accelerators of cutaneous wound healing (2), antioxidant agents (16) and stimulators of collagen synthesis in certain fibroblasts cells(3). Ferulic acid also promotes skin wound healing by accelerating epithelialization process (17, 18), and acts as antioxidant by inhibiting lipid peroxidation (17). The flavonoids have also antioxidant activity; the studies have showed that quercetin and rutin are protective for cutaneous neurovasculature cell populations subjected to oxidative damage (19). The presence of rutin in a dermatological formulation has stimulated skin wound healing by slowing down the lipid peroxidation, increasing catalase activity and decreasing protein carbonyl content (20). The objective of this work is to introduce and evaluate an original and natural product with complex therapeutic action in the spectrum of the medicinal preparations for burns and wounds. A complex formula based of a mixture of nine medicinal plants has been designed for the achievement of an ointment with good wound healing activity. The ointment formulation includes olive oil extract from the plant mixture, other natural ingredients with proved wound healing activity, respectively: sea buckthorn oil (Hippophae oleum), lavender essential oil (Lavandulae aetheroleum) and as thickening agents, coconut oil (Cocos oleum), beeswax (Ceraflava) and conifer resin (Resinapini). The plants from the mixture have been selected based on their wound healing capacity: Calendula officinalis L. (pot marigold, Asteraceae), Matricariachamomilla L. (chamomile, Asteraceae), Symphytum officinale L. (comfrey, Boraginaceae), Hypericum perforatum L. (St. John’s wort, Hypericaceae), Achillea millefolium L. (common yarrow, Asteraceae), Arctium lappa L. (burdock, Asteraceae), Plantago major L. (greater plantain, Plantaginaceae), Althaea officinalis L. (marshmallow, Malvaceae), Quercus robur L. (oak bark, Fabaceae). Ethanolic extracts from the plant mixture and ethanolic re-extracts from oil plant mixture extract and from ointment have been obtained and analyzed for polyphenols content, antioxidant activity and their influence on fibroblasts viability.

2. Material and methods

2.1. Reagents Eagle’s Minimal Essential Medium (MEM), natrium chloride, kalium chloride, disodium phosphate, potassium acid phosphate, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red), calcium chloride, penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), formic aldehyde (Fluka), acetic acid (Merck), ethanol (Chemical Company, Romania), fetal calf serum and neomycin (Biochrom), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, rutin hydrate, gallic acid monohydrate (Sigma), caffeic acid, chlorogenic acid, acid ferulic, catechin hydrate (Aldrich), sodium bicarbonate (Reactivul, Bucharest, Romania), aluminum chloride hexahydrate, methanol, potassium acetate (Scharlau, Spain), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Sigma).
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(Trolox), quercetin dihydrate (Fluka), salicin (Wako, Japan), olive-pomace oil (Olivarum oleum, Salvadori, Italy), sea buckthorn fruits oil (Hippophae oleum, SC Hofigal SA, Bucharest, Romania), organic coconut oil (Cocos oleum), lavender essential oil (Lavandulaeatheroleum), beeswax (Ceraflava), conifer resin (Resinapini) and medicinal plants: Calendula officinalis, Matricariachamomilla, Symphytum officinale, Hypericumperforatum, Achilleamillefolium, Arctiumlappa, Plantago major, Althaea officinalis, Quercusrobur (SC Santo Raphael SRL, Bucharest, Romania).

2.2. Apparatus Shimadzu UV-Vis spectrophotometer, UV mini 1240; Varian 310 – MS LC/MS/MS triple quadrupole mass spectrometer fitted with an electrospray ionization interface (ESI), ultrasonic cleaning bath ELSA-MATANTECH (60 Hz frequency, 750 W power). WTWInolab 720 pH-meter, Hettich MIKRO 120 centrifuge, laboratory orbital shaker Rotamax 120, Heidolph (Germany), Mithras LB 940 Multimode Microplate Reader from Berthold Technologies (Germany), inverted research microscope AXIO-Observer D1, Zeiss (Germany), vacuum rotary evaporator Laborata 4000, Heidolph (Germany).

2.3. Extracts preparation Alcoholic extracts were obtained in ethanol 70% using the following methods: maceration at room temperature with occasionally stirring for 7 days and with continuous stirring for 3 hours respectively; reflux for 3 h and for 30 minutes respectively. Plants mixture was grounded, then each 10 g of powder were submitted to extraction and the vegetable material /solvent ratio was 1:10 (w/v). Extracts were filtered through filter paper.

To prepare oil extract, 30 g of powdered vegetable mixture and 90 g olive oil were kept for 6 hours at 60 ºC in a thermostatic water bath, left over night at room temperature and then filtered through sterile cheesecloth. Sample for analyses of oil extract was prepared by alcoholic reextraction in a separatory funnel (20 mL oil extract and 60 mL ethanol 70%, v/v). After shaking the funnel vigorously for 15 min., the resulting emulsion was centrifuged at 3500 rpm for 7 min. Supernatants were collected and filtered through filter paper.

Sample for analyses of ointment was prepared as follows: a portion weighed of the total amount of ointment was extracted with ethanol 70% (v/v) by ultrasounds assisted extraction (15 min); the ointment /solvent ratio was 1:20 (w/v). The resulting extract was centrifuged and filtered in the same conditions as above. All extracts were kept at 4 ºC till further analyses.

2.4. Ointment preparation Plants mixture consists of 20 g of each of marigold flowers, St. John's Wort herba, comfrey leaves, burdock leaves, marshmallow leaves, 30 g of each chamomile flowers and plantain leaves and 15 g of each yarrow herba and oak bark, according to Romanian patent request A/00029/2012 (21).

190 g of dry, powdered plants mixture was extracted in 800 mL olive oil in a vessel equipped with mixing elements. Mixed olive oil with herbal powders was kept for 6 hours at 80 ºC in a thermostatic water bath, then the oil extract was filtered through sterile cheesecloth. For ointment preparation, filtered hot oil was first mixed with approx. 100 g shredded beeswax and 100 g pine resin to dissolve them, then 100 ml coconut oil and100 ml sea buckthorn oil (as the preparation temperature drops to keep unaltered the active ingredients) and finally 5 ml volatile oil of lavender. The mixture was continuous homogenized until completely cooled to room temperature.

2.5. Fibroblast bioassay Cells from mouse fibroblast cell line NCTC clone 929 (European Collection of Cell Cultures, Sigma-Aldrich, USA) were seeded into 24 well plates to form a sub-confluent monolayer at a density of 2x10^4 cells per well in Eagle’s Minimal Essential Medium (MEM) containing 10% fetal calf serum (FCS) and 1% antibiotics (penicillin/neomycin/streptomycin) and maintained at 37ºC in a humidified incubator of 5%
CO₂: 95% air atmosphere. After 24 h the culture medium was removed by aspiration. Dilutions from two tested vegetal extracts were made in MEM medium to give a range of final concentrations in the wells from 0.1 µg/mL to 50 µg/mL. The assay was conducted in triplicate for each sample concentration. Control cultures, received normal medium without herb extract. The cells were incubated and assayed for cell growth at 24 h, 48 h and 72 h. The test samples were the ethanolic extract and the oil extract which was prepared for in vitro analysis by ethanolic reextraction. These two extracts were initially concentrated under a vacuum rotary evaporator at 40°C, solubilized in water and diluted in MEM medium to give a stock concentration of 50 µg/ml and filtered through 0.2 µm Millipore sterile filter prior to addition to the cells.

The effect of the herbal extracts on the fibroblasts cell growth was assessed according to neutral red (NR) method described by Borenfreund and Purner (1984) with some modifications (22). The NR assay is used to measure cell viability. It has been used as an indicator of cytotoxicity in fibroblast cultures (23) and on other cell lines (24, 25). This simple and sensitive procedure is based on morphological and spectrophotometric criteria. Both assays were carried out on the same cell culture in a 24 well microtiter test plate. After the appropriate time interval the plates were transferred to an inverted microscope for any visible sign of morphological alteration (growth inhibition, vacuolization, rounding, detachment and lysis). The quantitative spectrophotometric procedure is based on the incorporation of NR, a supravital dye, into the lysosomes of viable cells. The medium was removed by aspiration and the cells washed with phosphate buffered saline, 0.5 mL freshly prepared neutral red (NR) solution, 5µL NR (50 µg/mL) in 495 µl MEM medium, was added to each well and the cells were incubated at 37°C for 3h. The NR solution was removed, and the cells washed rapidly with 1% CH₃O/1% CaCl₂ to remove unincorporated NR. To extract the dye from the lysosomes, 1% (v/v) acetic acid and 50% (v/v) ethanol was added to each well and the plated were shaken for 10 - 15 min at RT on an orbital shaker. Plates were transferred to a microplate reader and the optical density measured at 540 nm. Experiments for cell cultures were done in triplicate for each sample and data was reported as mean ± SD, calculated with Excel for Windows. Pair comparison of control and each sample was carried out by student t-test. Significant statistical differences were considered p<0.05.

2.6. LC-MS analysis of ethanolic extracts. The characterization of polyphenols in extracts was performed with liquid chromatography coupled with mass spectrometry (LC-MS) (26, 27).

Chemical characterization was carried out by using an Alltech ALTTIMA C18 5U column (100 x 3.2 mm, 5µm particle size). The mobile phase was methanol: bidistilled water = 70: 30 (v/v) and flow rate 0.6 mL/min, in an isocratic elution. The injection volume was 20 µL. Air was used as a drying gas at a pressure of 19 psi and 250 °C. The nebulizing gas was nitrogen to 40 psi and the capillary voltage had been established to the potential -4500V for negative ionization. The deprotonated molecular ion obtained was selected by the first quadrupole and then fragmented by collision with an inert gas (argon) to 1.5 mTorr pressure into the second quadrupole. Fragments were analyzed by the third quadrupole. Prior to these experiments, the tuning of mass spectrometer was performed using PPG both for positive and negative.

Gallic acid (GA), caffeic acid (CA), chlorogenic acid (ChA), ferulic acid (FA), salicin (S), (+)-catechin (C), quercetin (Q), rutin (R) were used as standards. 1 mL of each 0.1 mg/mL methanolic stock solution of mentioned standards were mixed and brought to 25 mL with methanol in a volumetric flask. Thus the concentration for each standard in the mixture was 4 µg/mL.
The concentration of standard in extract was calculated with the formula (1):

\[
\frac{A_{\text{standard}}}{C_{\text{standard}}} = \frac{A_{\text{sample}}}{C_{\text{sample}}} \tag{1}
\]

where: \(A_{\text{standard}}\) is integrated area of the peak for each standard; \(C_{\text{standard}}\) is the known concentration of standard; \(A_{\text{sample}}\) is the integrated area of the peak for each extract; \(C_{\text{sample}}\) concentration of standard determined in extract.

Each sample was performed in triplicate and SD values were given. Results were expressed as mg standard/100 g dw.

2.7. Total phenols and flavonoids determinations Total phenols (TP) content was determined using the Folin-Ciocalteu method and working procedure was described in our previous works (28, 29). Absorbance (A) was measured at wavelength of 761 nm. Calibration curves absorbances vs. antioxidant concentration were drawn. Equations, \(r^2 / n\) (correlation coefficients / number of determinations) and linearity domains for obtained calibration curves were:

\[
A = 0.0037 \times \text{caffeic acid concentration} - 0.0626, \quad r^2 / n = 0.9933 / 11 \quad \text{(linearity domain = 30-200 mg/L)}; \tag{2}
\]

\[
A = 0.0018 \times \text{chlorogenic acid concentration} - 0.0438, \quad r^2 / n = 0.9956 / 10 \quad \text{(linearity domain = 40-200 mg/L)} \tag{3}
\]

Total flavonoid content of extracts was measured colorimetrically using aluminium chloride method and absorbance was recorded at \(\lambda = 425\) nm. Working procedure was described in our previous works (28). Equation, the correlation coefficient \((r^2)\) and linearity domain obtained for the calibration curve absorbance as a function of concentration of rutin was:

\[
A = 0.0029 \times \text{rutin concentrations} + 0.0103, \quad r^2 / n = 0.9933 / 11 \quad \text{(linearity domain = 30-200 mg/L)} \tag{4}
\]

Concentrations of samples were determined from equations of calibration curves over linear range and final results were expressed as mg standard equivalents /100g dry weight (dw) for all extracts. All determinations were made in triplicate and SD values were given.

2.8. DPPH free radical scavenging activity DPPH free radical scavenging activity was made according to our previous work (29). Thus, 500 µL standard/sample were mixed with 500 µL DPPH solution (0.135 mM in 70% (v/v) ethanol) and the mixture kept in dark for 30 minutes. Absorbance was measured at the wavelength of 525 nm. Calibration curve inhibition percent (% I) vs. concentration of TROLOX was drawn. The equation curve, correlation coefficients \((r^2)\) and linearity domain obtained were:

\[
\% I = 0.9455 \times \text{Trolox concentration} + 47.5, \quad r^2 / n = 0.9902 / 7 \quad \text{(linearity domain = 2.5-25 μmoles/L trolox)} \tag{5}
\]

From equation of calibration curve was determined the ability of free DPPH radical scavenging of antioxidant compounds from samples. Antioxidant capacity of samples was expressed as mmolestrolox equivalents (TE)/L sample. All determinations were made in triplicate; SD values were given.

2.9. Stability testing of formulated ointment Centrifuge test. 1 gram of ointment was centrifuged for 15 min at 3000 rpm at room temperature at 24 hours after preparation. Then
the ointment was homogenized by vortex for 30 seconds and centrifuged again for other 15 min. Phase separation was evaluated before the ointment was homogenized at vortex and after the second centrifugation (30).

**pH ointment determination.** pH values of the ointment were obtained by direct potentiometric method and according to Bucur (31). Thus, 6 ml of distilled water warmed at 80 °C was poured over 1 gram of ointment and shaken vigorously for 1 minute, then filtered. The filtrate was brought to room temperature and pH was measured.

3. Results and discussions

3.1. Fibroblast bioassay

The morphological modifications of fibroblast culture treated with ethanolic extract and oil re-extract were examined microscopically for all concentrations and at all intervals of time selected. No alteration sign was observed for all samples.

The results of spectrophotometric determinations are shown in figure 1. Absorbance is proportional to the numbers of cells with intact membrane. Figure 1a and 1b shows the fibroblasts viability in the presence of ethanolic and oil re-extract. The results of paired student t-test showed that there were no significant differences (p>0.05) between control and each concentration of extracts at all-time intervals suggesting that both extracts are not cytotoxic.

![Figure 1](image)

**Figure 1.** Cell viability of NCTC fibroblasts cultured at 37°C for 24, 48 and 72 h with different concentrations of ethanolic extract (a) and oil re-extract (b), analyzed by Neutral red assay. Control: concentration 0 µg/ml. statistic differences were not observed (p>0.05).

Figure 2 presents the fibroblasts cell morphology at 72 h after the addition of the extracts. The cells were stained with Hematoxylin – Eosin and photographed with a Zeiss Axio Observer optic microscope. The cytotoxic effect of the ethanol and oil re-extract on fibroblasts cell was not detected microscopically.

3.2. Chemical characterization of alcoholic extracts by LC-MS/MS analysis

The LC-MS method allowed the identification and quantification of the main phenolic compounds in plant mixture extracts. Compounds in extracts were identified by detailed studies of their MS-MS spectral data by comparing their spectra with those of the standards and with literature data. The results are presented in Table 1.
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Table 1. Quantitative LC-MS analysis of the alcoholic extracts and re-extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>GA (mg/100 g dw)</th>
<th>FA (mg/100 g dw)</th>
<th>CA (mg/100 g dw)</th>
<th>ChA (mg/100 g dw)</th>
<th>Q (mg/100 g dw)</th>
<th>R (mg/100 g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maceration 7 days</td>
<td>0.339 ± 0.10</td>
<td>0.122 ± 0.006</td>
<td>0.347 ± 0.063</td>
<td>5.95 ± 0.31</td>
<td>1.20 ± 0.22</td>
<td>2.73 ± 0.11</td>
</tr>
<tr>
<td>Maceration 3h</td>
<td>0.336 ± 0.041</td>
<td>0.161 ± 0.059</td>
<td>0.416 ± 0.089</td>
<td>11.9 ± 1.40</td>
<td>1.13 ± 0.15</td>
<td>3.57 ± 1.00</td>
</tr>
<tr>
<td>Reflux 3h</td>
<td>0.252 ± 0.031</td>
<td>0.142 ± 0.010</td>
<td>0.412 ± 0.014</td>
<td>3.62 ± 0.43</td>
<td>3.56 ± 0.93</td>
<td>2.76 ± 0.31</td>
</tr>
<tr>
<td>Reflux 30 min</td>
<td>0.278 ± 0.042</td>
<td>0.118 ± 0.017</td>
<td>0.433 ± 0.20</td>
<td>5.26 ± 0.26</td>
<td>2.99 ± 0.26</td>
<td>2.55 ± 0.27</td>
</tr>
<tr>
<td>Oil re-extraction</td>
<td>2.43 ± 0.44</td>
<td>0.561 ± 0.16</td>
<td>1.26 ± 0.11</td>
<td>12.2 ± 2.3</td>
<td>1.42 ± 0.59</td>
<td>8.23 ± 2.7</td>
</tr>
<tr>
<td>Ointment re-extraction by US</td>
<td>6.98 ± 0.33</td>
<td>0.832 ± 0.047</td>
<td>1.76 ± 0.26</td>
<td>5.49 ± 0.11</td>
<td>-</td>
<td>7.29 ± 0.49</td>
</tr>
</tbody>
</table>

Re-extracts (µg/g)

<table>
<thead>
<tr>
<th>Sample</th>
<th>TE (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil re-extraction</td>
<td>13.5 ± 0.90</td>
</tr>
<tr>
<td>Ointment re-extraction by US</td>
<td>10.9 ± 1.2</td>
</tr>
</tbody>
</table>

Of all the standards used, gallic acid (GA), caffeic acid (CA), chlorogenic acid (ChA), ferulic acid (FA), quercetin (Q) and rutin (R) were identified and quantified in the analyzed extracts. Results of LC-MS analysis are shown in Table 2. Of all compounds measured, chlorogenic acid was found in highest amounts, followed by rutin and quercetin and in smaller amounts by caffeic acid, gallic acid and ferulic acid in ethanolic extracts. As for re-extracts, values for polyphenols and flavonoids are smaller because smaller amounts of substances are extracted in oil. Values for some compounds are higher for cream re-extract, this happens probably due to the presence of the other ingredients of cream.

3.3. Total phenols and flavonoids content determination. DPPH radical scavenging activity of extracts Results are in concordance with the ones obtained by LC-MS analysis and are presented in Table 2.

Table 2. Total phenols content, flavonoids content and antioxidant activity of the extracts from the plant mixture and of the re-extracts of oil extract and cream

<table>
<thead>
<tr>
<th>Sample</th>
<th>TP CAE (g/100 g dw)</th>
<th>TP ChAE (g/100 g dw)</th>
<th>FL RE (g/100 g dw)</th>
<th>DPPH TE (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maceration 7z</td>
<td>1.77 ± 0.14</td>
<td>3.26 ± 0.28</td>
<td>1.54 ± 0.038</td>
<td>12.1 ± 1.3</td>
</tr>
<tr>
<td>Maceration 3h</td>
<td>2.51 ± 0.027</td>
<td>4.76 ± 0.055</td>
<td>1.53 ± 0.14</td>
<td>12.4 ± 1.7</td>
</tr>
<tr>
<td>Reflux 3h</td>
<td>3.63 ± 0.32</td>
<td>6.65 ± 0.65</td>
<td>0.915 ± 0.088</td>
<td>10.9 ± 1.2</td>
</tr>
<tr>
<td>Reflux 30 min</td>
<td>4.55 ± 0.36</td>
<td>8.55 ± 0.74</td>
<td>0.921 ± 0.086</td>
<td>13.5 ± 0.90</td>
</tr>
<tr>
<td>Oil re-extraction</td>
<td>0.728 ± 0.11</td>
<td>13.5 ± 0.23</td>
<td>4.90 ± 0.53</td>
<td>1.10 ± 0.059</td>
</tr>
<tr>
<td>Ointment re-extraction by US</td>
<td>2.77 ± 1.7</td>
<td>52 ± 3.4</td>
<td>41.6 ± 4.7</td>
<td>-</td>
</tr>
</tbody>
</table>

The amount of polyphenols is twice higher when expressed as chlorogenic acid compared to caffeic acid equivalents. The results showed that extracts contain good amounts of polyphenols like caffeic, chlorogenic, gallic, and ferulic acids, as well as quercetin and rutin, compounds reported in the literature as having good wound healing activity. These amounts determine and confirm the wound healing and epithelialization potential of the ointment.

*Antioxidant activity of extracts* evaluated by DPPH free radical scavenging assay is illustrated in Table 2. The increased activity of DPPH radical scavenge for *alcoholic extracts* indicates high antioxidant activity of extracts and also confirms the wound healing potential of the ointment. Moreover, the antioxidant capacity of the various ingredients from the ointment has been highlighted by numerous data from literature (32-35). For *oil re-extracts* the ability of DPPH radical scavenge has very small values and not detectable in case of cream re-extract.

### 3.4. Stability testing of formulated ointment

**Centrifuge test.** No phase separation was observed after 30 (2 x 15) minutes centrifugation. **Appearance.** Samples were semisolid, pale green, leaving a homogenous smear on the glass plate. No phase separation was noticed during 3 months of storage at different temperatures (5 °C, 22 °C).

**pH (acidity) ointment determination** The pH value of the ointment obtained by direct potentiometric method for cream at different temperatures (5 °C, 27 °C and 45 °C) was 4.2. When applied the method from (31) the pH value obtained for the filtrate was 4.3. According to some literature data (36, 37) local acidification of the wound (by ointment pH around 4, as in the case of honey for example) promotes healing of the tissues by preventing appearance of non-ionized histotoxic form of ammonia, formed by the action of urease (from urease-producing microorganisms) on urea in extracellular fluid. In an acidic medium, ammonia (NH₃) is converted to ionized, nontoxic, ammonium ion (NH₄⁺) (37). In addition, acidification of the wound increases oxygen intake and pO₂ on wound surface by increasing oxyhemoglobin-hemoglobin dissociation, due to an appropriate shift in the oxyhemoglobin-hemoglobin dissociation curve (Bohr effect).

### 3.5. Case reports

The effectiveness of the ointment has been clinically highlighted by some case reports, both acute (mainly burns) and chronic wounds, with the informed consent obtained from the patients. First case represents an acute wound – scald injury IIA-IIB degree on the dorsum of the left hand (Figure 3). Wound characteristics were: moist, pink-whitish, intermediate depth lesion, moderate and serous secretion, shiny portions indicating plasmorrhagia (deeper areas). After painless treatment with the ointment, the patient achieved substantial improvement only 4 days later, involving two dressing changes, with beginning of epithelization and blurring of the shiny areas. The wound was almost fully epithelized after 10 days.

![Initial burn wound](image1)
![After 4 days of treatment](image2)
![After 2 weeks of treatment](image3)

*Figure 3.* Results obtained after applying the ointment on a scald wound IIA-IIB degree
The second case is a chronic wound – leg ulcer, distal third of the right leg, arterial-venous etiology, dry eschar, and little secretion (Figure 4). The leg ulcer was 1 year old and completely resistant to other usual treatments. After only 12 days, dressing changes to 1-2 days, a remarkable evolution was observed, with clear wound contraction and highly progressive epithelization.

![Figure 4. Effect of the ointment on a leg ulcer](image)

4. Conclusions

Given the increasing interest for preparations of natural origin, the present ointment provides a topical composition for the treatment of burns and wounds, which is effective, stable, pleasant, relatively inexpensive, easily managed, safely applied and used. The beneficial properties result by adding the therapeutic effects of oil extracts from a variety of medicinal plants to those of a lipophilic ointment base consisting of vegetables oils, beeswax, pine resin and lavender essential oil, all of which exhibiting real therapeutic value and importance, which have been confirmed by a plethora of data from literature. Further in vivo and clinical studies are needed to assess the entire range of ointment properties, the best indications and modalities of administration in practice.

References


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