Experimental Research of an Innovative Ointment with Increased Wound Healing Activity - DERMAPLANT -

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Disclosure

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Wound healing

- involves a complex, dynamic process
- 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 may cause cellular damage by different mechanisms such as peroxidation of membrane lipids
- This suggests that the presence of the antioxidants may be favorable for healing

Medicinal plants and natural remedies

- have been used with confidence by mankind from ancient times to treat various skin diseases
- their application for wound healing is actually supported by many scientific studies
- many advantages: cheap, affordable, effective, easy to manage, also safe as side effects are rare and minor, mainly local hypersensitivity
- contribute to wound healing and tissue regeneration by multiple mechanisms
- bioactive compounds – e.g. polyphenols

The objective of this work

- preclinical study of an original complex formula, made solely on the basis of medicinal plants and natural ingredients, and conditioned as an ointment to stimulate proper wound healing, which was called DERMAPLANT
- complex formula based of a mixture of nine medicinal plants, together with other natural ingredients with known wound healing activity.

The ointment formulation

- olive oil extract from the plant mixture
- sea buckthorn oil (*Hippophae oleum)*
- coconut oil (*Cocos oleum)*
- beeswax *(Cera flava)*
- conifer resin *(Resina pini)*
- lavender essential oil *(Lavandulae aetheroleum)*.

The plants from the mixture

- *Calendula officinalis* L. (pot marigold)
- *Matricaria chamomilla* L. (chamomile)
- *Symphytum officinale* L. (comfrey)
- *Hypericum perforatum* L. (St. John's wort)
- *Achillea millefolium* L. (common yarrow)
- *Arctium lappa* L. (burdock)
- *Plantago major* L. (greater plantain)
- *Althaea officinalis* L. (marshmallow)
- *Quercus robur* L. (oak bark).

Ethanolic extracts

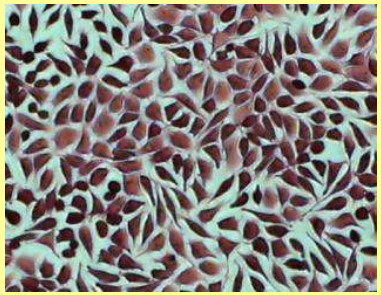
- In a previous study, ethanolic extracts from the plant mixture and ethanolic re-extracts from oil plant mixture extract and from ointment have been obtained and analyzed for:
  - polyphenols content – by **LC-MS analysis**
  - antioxidant activity – by **DPPH free radical scavenging assay**
  - their influence on fibroblasts viability – by **Neutral Red assay**

Fibroblast bioassay

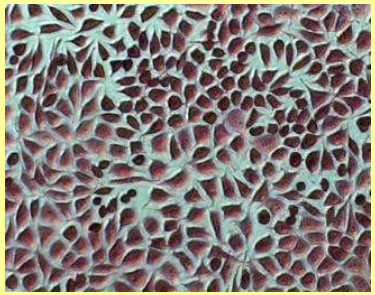
- The morphological modifications of fibroblast culture treated with ethanolic extract and oil re-extract were examined microscopically.
- spectrophotometric determinations
- No alteration sign was observed for all samples.
- there were no significant differences ($p>0.05$) between control and each concentration of extracts at all time intervals suggesting that both extracts are not cytotoxic.

Fibroblasts cell morphology at 72 hours after the addition of the extract

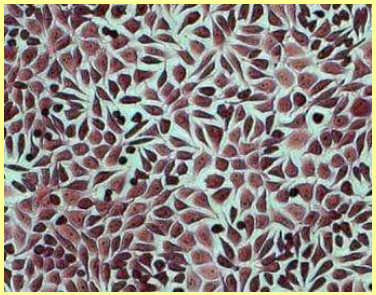
Control



Ethanolic extract 2.5 µg/ml



Oil re-extract 0.05 µg/ml



Total phenols and flavonoids content determination

extracts contain good amounts of polyphenols like caffeic, chlorogenic, gallic and ferulic acids, as well as quercetin and rutin, compounds reported in literature as having good wound healing activity

Antioxidant activity of extracts

- evaluated by DPPH free radical scavenging assay
- The increased activity of DPPH radical scavenge for **alcoholic extracts** indicates high antioxidant activity of extracts
- Moreover, the antioxidant capacity of the various ingredients from the ointment has been highlighted by numerous data from literature

Material and methods

- The active ingredient is the oily extract of the plant mixture, and the conditioned form is an ointment.
- Experimental data will allow preclinical pharmacological evaluation of the product, tested both as an extract and as an conditioned product (ointment), in order to establish the basic safety and efficacy aspects of the product.

In vitro and in vivo experimental models

- **Product safety tests:**
  - culture of reconstituted dermis, in vitro experimental model, for assessing irritant or corrosive activity on the epidermis;
  - in vitro pyrogenicity test: bacterial endotoxin (LAL)
  - microbial load test
- **Efficacy tests:**
  - test for sensitizing potential (in vivo);
  - in vitro wound repair test;
  - healing of thermal wounds (in vivo)

1. Testing irritant-corrosive action on a reconstituted dermis model

- **Evaluation of dermal toxicity**
- **Test protocol**: Work was carried out in accordance with the testing protocol provided by the kit supplier, Mattek, a distributor of in vitro kit from the American company Mattek. Protocol: In Vitro EpiDerm ™ Skin Irritation Test (EPI-200-SIT)

1. Evaluation of dermal toxicity

- **Material and methods**
- Test kit (human reconstituted dermis), delivered as viable cultures, 24 human reconstituted dermal inserts in a 24-well plate, inserted into agarose gels and supplemented with culture medium.
- We have performed viability tests, using the EPI-SKIN model, on the product "Active Product - Dermaplant Extract“ and "Therapeutic Product - Dermaplant Ointment" in three independent replicate series of 3 sets of determinations in each independent replicate.

1. Evaluation of dermal toxicity

- The data recorded after the test:

Product	Mean DO	Stdev	p(2)	Relative viability
PBS*,1	1.887	0.2703	-	100%
SDS**	0.031	0.0175	-	0
Dermaplant extract	1.413	0.4634	0.035	69.88%
Dermaplant ointment	1.328	0.6436	0.035	74.46%

- * The average of three independent replicas
- ** Positive control (sodium dodecylsulfate); 1: negative control; 2: student t test, 2 queues, homoscedical dispositions versus positive control

1. Evaluation of dermal toxicity

- According to the EU and GHS classification (category R38 / Category 2 or unmarked) an irritant is predicted if the tissue viability of three individual tissues exposed to the test substance is below 50% the relative viability of the negative controls.

In vivo result	In vivo prediction
Medium tissue viability ≤50%	Irritant (I), (R38 or GHS 2 category)
Medium tissue viability >50%	Non-irritant (NI)

- According to the classification criteria, it is concluded that the products "Active Product – Dermaplant Extract" and "Therapeutic Product - Dermaplant Ointment" are non-irritating (NI), and, in conclusion, can be used safely, according to the instructions of use.

2. Evaluation of endotoxin load - LAL method - gel-clot technique

- The bacterial endotoxin (BET) assay is used to detect endotoxins in gram negative bacteria using the amoebic lysate from Limulus polyphemus or Tachypleus tridentatus.
- The technique used in the microbiology lab for the detection of bacterial endotoxins is LONZA gel-clot LAL.
- The gel-clot method is based on the opacifying and gelling of the LAL extract in the presence of bacterial endotoxins.
- Control of bacterial endotoxins (LAL test - gel-clot method) provides an in vitro alternative to the rabbit pyrogenicity test.
- **The LAL reagent** used in the test is a lyophilized product obtained from *Limulus polyphemus* amoebic lysate. The LAL reagent is extemporaneously reconstituted with LAL water by gentle rotation.

2. Evaluation of endotoxin load - LAL method - gel-clot technique

- The endotoxin load was estimated by the LAL test for the DERMAPLANT extract because the ointment product may eventually be contaminated with endotoxins from plants used in the manufacturing process.
- The ointment has characteristics that make it impossible to apply the endotoxin test.
- According to the data obtained, the DERMAPLANT - Extract product was found to be within the recommended endotoxin content limits for topical products and medical devices type II, respectively below 0.5 EU / mL.

3. Method of testing microbial contamination

- **Cultivated media used**:
  - A (casein soya bean digest broth)
  - B (Casein soya bean digest agar)
  - C (Sabouraud-glucose agar with chloramphenicol)
  - peptone water buffer solution with sodium chloride, pH = 7
  - E (Enterobacteria enrichment broth-Mossel)
  - D (Lactose monohydrate broth)
  - F (Crystal violet, neutral red, bile agar with glucose)
  - N (Cetrimid agar)
  - O (Baird-Parker agar)
  - I (Tetrationat bile brilliant green broth)
  - K (XLD agar).
- **Testing methodology**: total number of viable aerobic microorganisms - in-depth inoculation method, 1/10 dilution

3. Method of testing microbial contamination

- DERMAPLANT Extract and DERMAPLANT Ointment Product were used for the determination of bacterial, fungal and yeast contaminants, according to the described protocol.
- Following the evaluation, no contaminants were found in the pathogenic microorganisms category.
- At the same time, although it has presented a microbial charge of non-pathogenic species, the product in its current form is not yet suitable for medical devices coming into contact with injured tissues, for which a new sterilization operation is recommended, most likely by irradiation, which would ensure the recommended sterility level for products used in burns treatment.

4. Determining the cells viability

- Mouse embryonic cells cultures 3T3 - NIH/3T3 (ATCC CRL-1658)
- Cells were inoculated at a density of $5x10^3$ cells/cm^2 in 96-well plates and were precultivated for 24 hours at 37 ° C and 5% CO_2 in DMEM-F12 medium supplemented with 10% fetal bovine serum.
- After 24 hours, the medium is mixed with fresh medium, in which serial dilutions of the test product (dilution interval 10^{-3} - 10^{-5}) were made.
- In the case of the ointment, solubilization was done in DMSO (1:10), followed by dilution of the solution in the working medium.

4. Determining the cells viability

- After 72 hours of incubation, cells were aspirated, washed with working medium (2 washes), followed by incubation of cells with MTS reagent (3 hours) in working environment, and determination of cell viability.
- Dermaplant Extract demonstrated a remarkable proliferation-stimulating capacity (+ 18% increase in proliferation rate).

5. Testing the ability to stimulate the healing of burned wounds

- Testing was performed using the rat model. Worked on white male Wistar rats, 180-200 g, provided by the Cantacuzino Institute biobase, Bucharest, Romania.
- Two variants of the test model:
  - the conventional model in which the lesion is performed on both sides of the animal
  - a modified model in which the thermal injury is achieved on a single flank;
- a “control” group of animals, where the lesion is not treated
- a “treated“ group in which the test preparation is applied.

5. Testing the ability to stimulate the healing of burned wounds

- In both cases, the burned wounds were obtained by applying a dorsal shaved skin exposure at a temperature of 90 °C for 10 seconds on anesthetized animals with ketamine (50 mg/kg, ip).
- After this exposure, the animals were allowed to rest for 24 hours, after which they were treated.

5. Testing the ability to stimulate the healing of burned wounds

- The following groups were set up:

No	Designation	Remark
1	Treated group	Bilateral thermal lesion
2	Control group	Unilateral thermal lesion
3	Untreated group 2	Unilateral thermal lesion

- The groups were treated every 48 hours for 6 days.

5. Testing the ability to stimulate the healing of burned wounds

- Results of experiments - visual observations of tissue regeneration

No	Designation	Remark	Result (% lesion area)
1	Treated group	Bilateral thermal lesion – control area	52%
		Bilateral thermal lesion – treated area	41%
2	Control group	Unilateral thermal lesion	31%
3	Treated group 2	Unilateral thermal lesion	56%

5. Testing the ability to stimulate the healing of burned wounds

- From the results analysis, it can be deduced that in the case of bilaterally injured animals, there is probably an influence (mediated by the production of cytokines and lymphokines), which leads to an improved repair on the untreated flank.
- On the "unilateral" model there is a net stimulation of the repair process in the treated animals, compared to control.
- To elucidate these aspects, it is proposed to continue the research by using multiplex kits to monitor the effect of the product on the production of cytokines and lymphokines involved in the inflammatory process and wound healing.

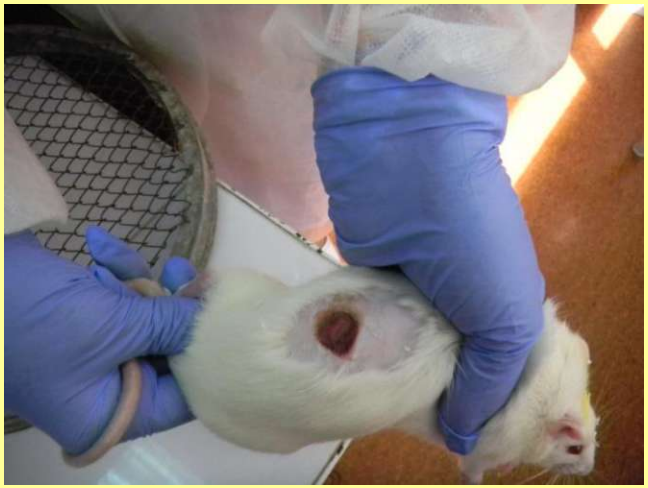
6. Determining the level of cytokines

- A more advanced re-epithelization of the treated animals compared to the control group was observed.
- The treatment consisted of the daily application of the ointment preparation on the surface of the wound.
- The wounds were produced by applying, on the first day of the experiment, a heated device at 80 ^{o}C, and keeping it in contact with the dermis for 10 seconds on the surface of the previously shaved skin of the animals.
- After injury, the treatment was performed.
- No occlusive dressing was used.

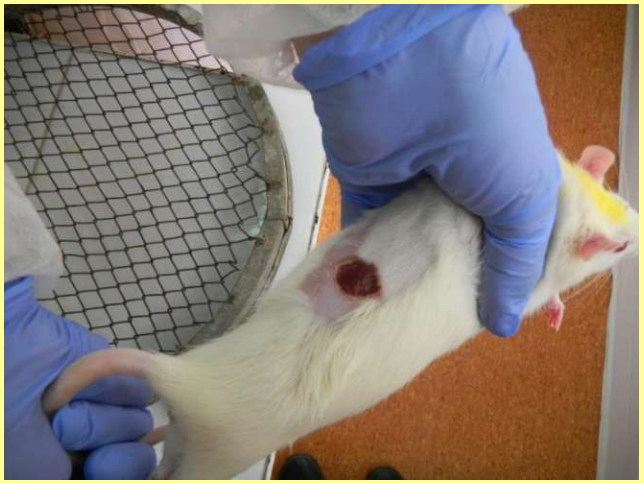
6. Determining the level of cytokines

- **First day**

Treated



Non-treated



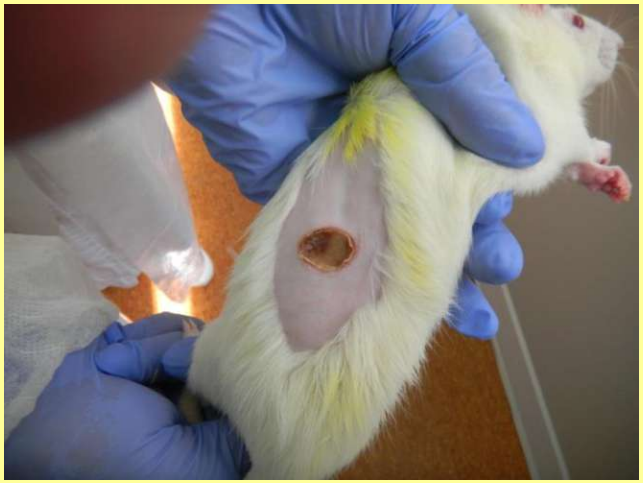
6. Determining the level of cytokines

- The treatment was continued for a period of 14 days, on days 2-4, 7-10, 13, 14.
- During the experiment, the animals were fed and received water ad libitum.
- No adverse effects due to treatment were observed.
- Although recovery of the control group was slower, no severe adverse effects or mortality were reported.

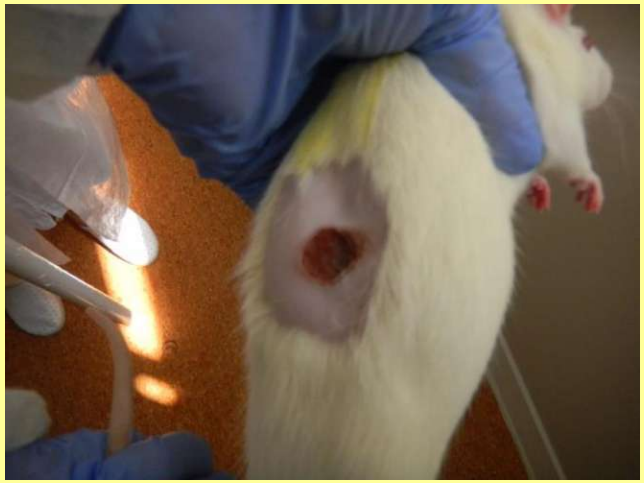
6. Determining the level of cytokines

- 7^{th} day

Treated



Non-treated

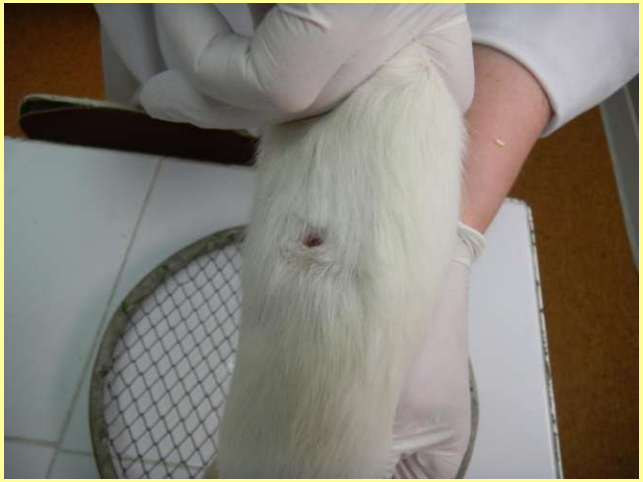


6. Determining the level of cytokines

- 14^{th} day

Treated

Non-treated

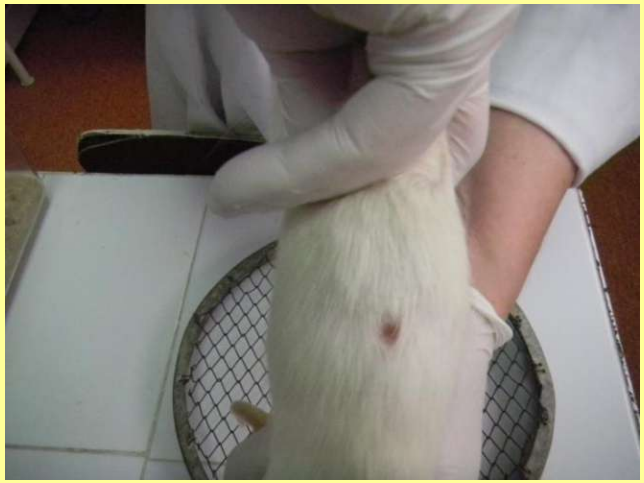


6. Determining the level of cytokines

- 14^{th} day

Treated

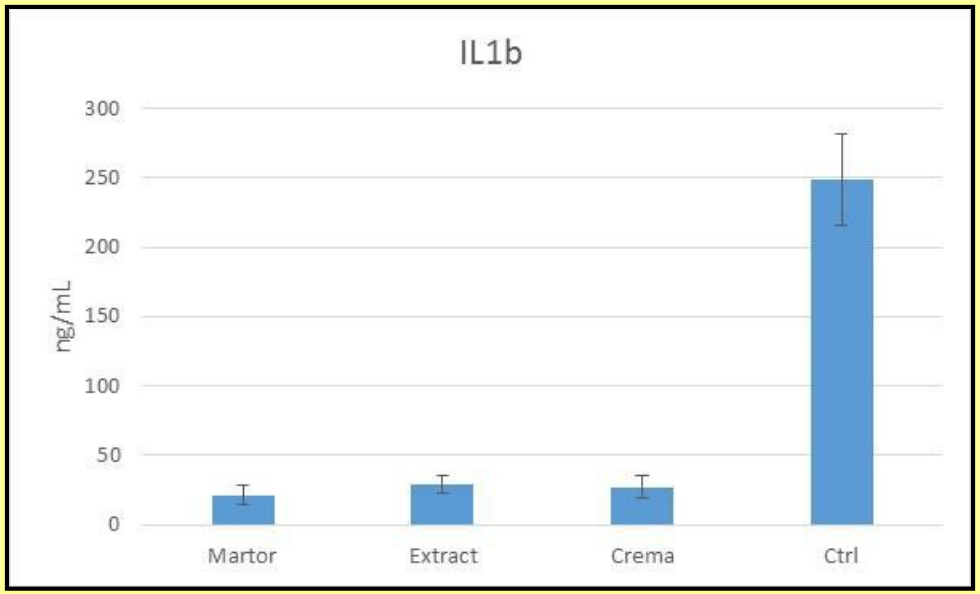
Non-treated



6. Determining the level of cytokines

- Cytokine levels - IL1β, TNFα and IL6 were determined in the culture supernatants collected after the ToxDerm kit experiment, using commercial ELISA kits, according to the manufacturer's instructions (ab214025, ab46087, ab46042, respectively) in triplicate.
- There is a significant reduction in the level of proinflammatory cytokines IL1β and TNFα, as well as a maintenance level comparable to the control for IL6.

6. Determining the level of cytokines



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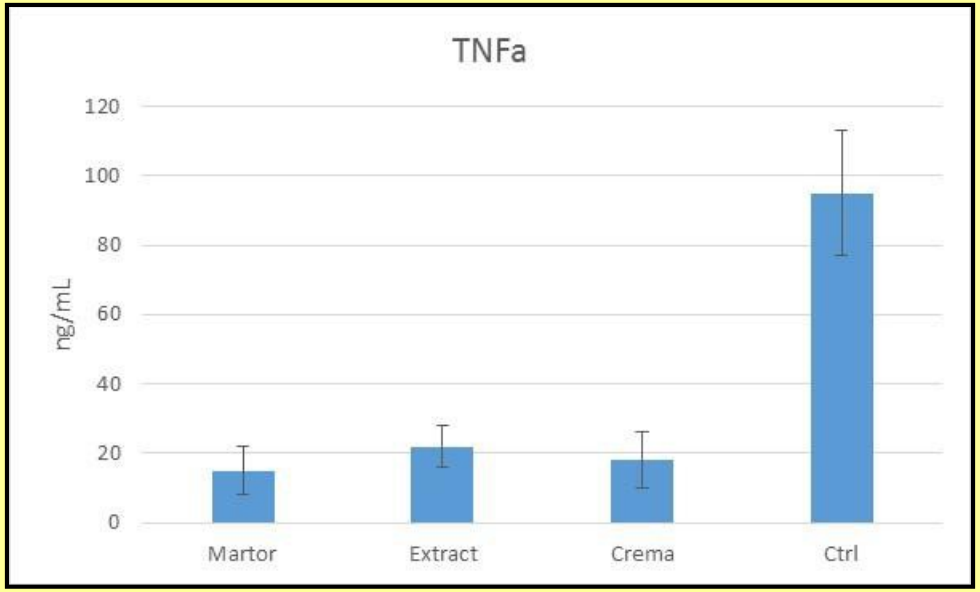
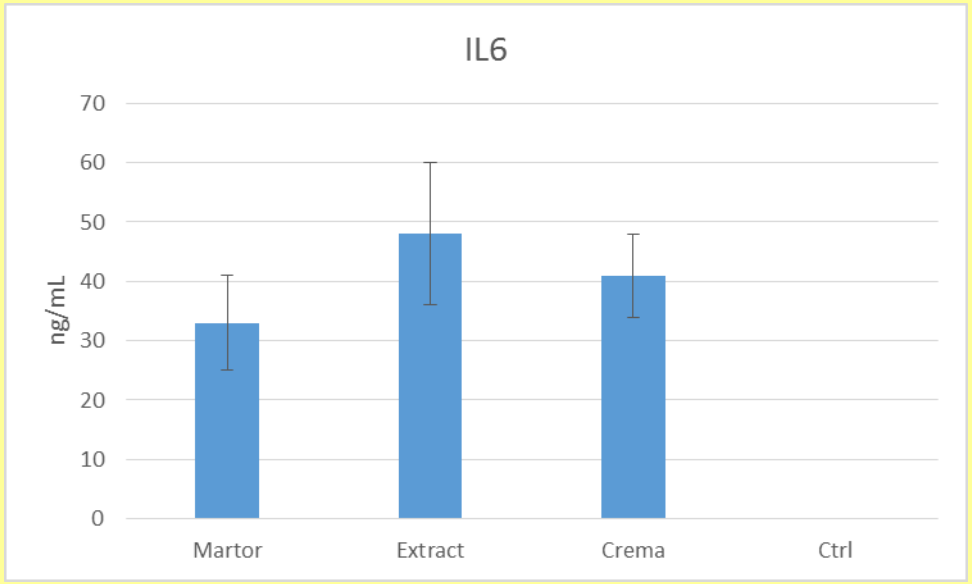


6. Determining the level of cytokines



7. Case reports

The effectiveness of the ointment

-clinically highlighted by some case reports:

- acute wounds (mainly burns)
- chronic wounds.

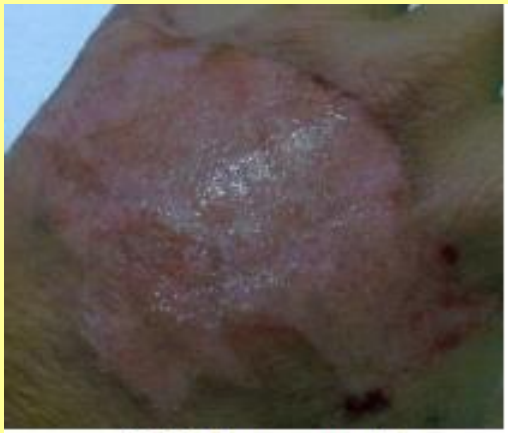
First case

- an acute wound
- scald injury IIA-IIB degree on the dorsum of the left hand
- Wound characteristics: moist, pink-whitish, intermediate depth lesion, moderate and serous secretion, shiny portions indicating plasmorrhagia (deeper areas).

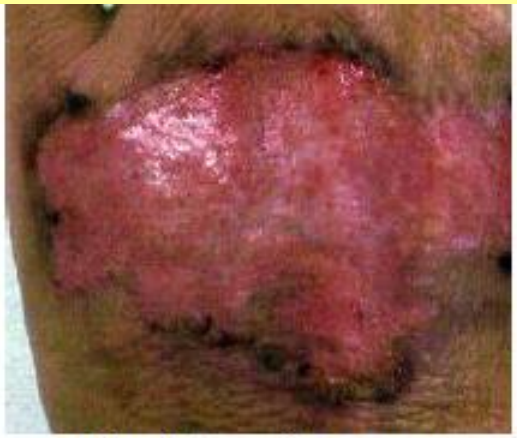
- painless treatment with the ointment
- substantial improvement only 4 days later, involving two dressing changes
- beginning of epithelization and blurring of the shiny areas.

The wound was almost fully epithelized after 10 days.

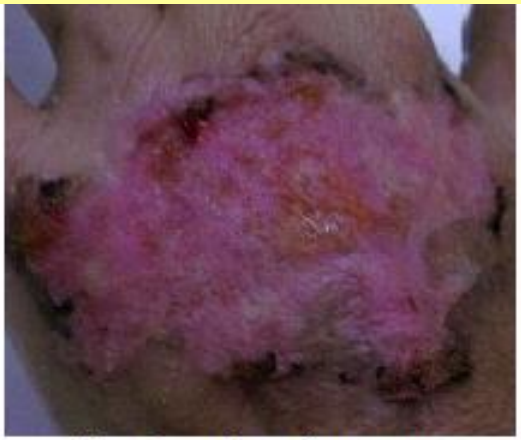
Results obtained after applying the ointment on a scald wound IIA-IIB degree



Initial burn wound



After 4 days of treatment



After 2 weeks of treatment

The second case

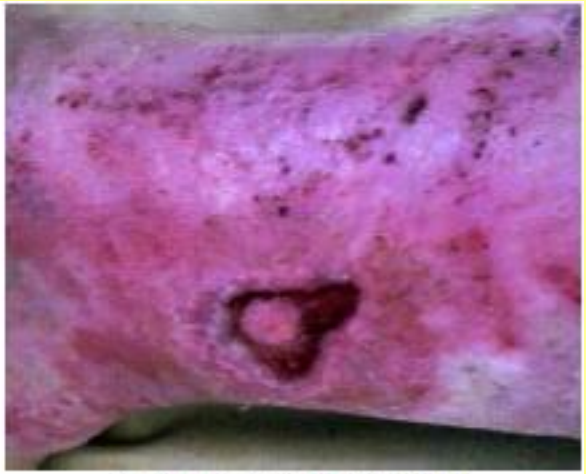
- a chronic wound
- leg ulcer, distal third of the right leg
- arterial venous etiology
- dry eschar, little secretion.

The leg ulcer was 1 year old and completely resistant to other usual treatments.

After only 12 days, dressing changed to 1-2 days, a remarkable evolution was observed, with clear wound contraction and highly progressive epithelization.

Effect of the ointment on a leg ulcer

Leg ulcer before treatment



After 12 days of treatment

Conclusions

1. The preparations do not show dermal toxicity, as demonstrated by the use of the In Vitro EpiDerm ™ Skin Irritation Test
2. Dermaplant Extract preparation, tested in vitro on 3T3 fibroblasts, demonstrated a remarkable proliferation-stimulating capacity (+ 18% increase in proliferation rate)
3. Safety tests have revealed that the product is free of endotoxin-like contaminants and is therefore safe for application to damaged skin.
4. There was no evidence of pathogenic germs, and the microbial load was within the limits imposed by Pharmacopoeia for topical preparations.

Conclusions

5. The evaluation of "in vivo" repair effect revealed a stimulation of wound healing on a model of unilateral thermal injury in the rat, the 6-day repair process being accelerated by 69%.
6. The product has good bioavailability, being fully absorbed in the examination 24 hours after application.
7. The anti-inflammatory and wound healing properties were highlighted by the decrease in the level of proinflammatory cytokines IL1β and TNFα .
8. Further research is needed to evaluate the clinical effects of the product and its ability to stimulate wound healing.

Thank you for your attention!