*wjpmr, 2022,8(3), 300 – 309.*

**WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH**

www.wjpmr.com

**SJIF Impact Factor: 5.922**

Research Article

ISSN 2455-3301

WJPMR

# EXPERIMENTAL SAFETY AND EFFICACY OF AN INNOVATIVE OINTMENT WITH WOUND HEALING EFFECT

***[1]Andrei Zbuchea, MD, PhD and [2]Radu Albulescu, PhD Biochem**

[1]Department of Plastic Surgery, District Emergency Hospital, 100, Gageni Street, Ploiesti, Romania.
[2]Research and Development – ICCF, 112, Calea Vitan, Bucharest, Romania.

***Corresponding Author: Andrei Zbuchea, MD, PhD**
Department of Plastic Surgery, District Emergency Hospital, 100, Gageni Street, Ploiesti, Romania.

**Article Received on 21/01/2022** **Article Revised on 11/02/2022** **Article Accepted on 01/03/2022**

## ABSTRACT

Within the therapeutic armamentarium for wound healing, this work aims to assess the qualities of an innovative natural preparation. The proposed ointment has a complex composition and is made exclusively from natural ingredients, which also has multiple actions to facilitate wound healing: extracts from nine medicinal plants (marigold, chamomile, comfrey, St. John's wort, yarrow, burdock, plantain, mallow and oak bark), vegetable oils (sunflower, coconut and sea buckthorn), beeswax, conifer resin and volatile lavender oil. The proposed ointment was experimentally investigated in vitro and in vivo, through a preclinical pharmacological evaluation of the product, to establish basic safety issues and effectiveness: reconstituted dermal culture, in vitro pyrogenicity test, bacterial load test, cells viability test, thermal wound healing (in vivo), and determining the level of cytokines. The preparations do not show dermal toxicity, are free of endotoxin-like contaminants and of pathogenic germs, and are therefore safe for application on injured skin. Dermaplant Extract, tested in vitro on 3T3 fibroblasts demonstrated a remarkable ability to stimulate proliferation (+ 18% increase in proliferation rate). The evaluation of the "in vivo" repair effect showed a stimulation of the repair of the damaged dermal tissue, on a model of unilateral thermal injury in rats, the repair process at 6 days being accelerated by 69%. The evaluation of the level of cytokines in the serum of treated animals highlighted significant reductions in the level of proinflammatory cytokines IL1β and TNFα.

## INTRODUCTION

Modern experimental research has shown the beneficial effects of herbal preparations in ameliorating (and even curing) wounds of various etiologies, but especially burn wounds, which are an important public health problem. Numerous researches are currently being carried out worldwide to discover new natural products and new pharmaceutical formulations dedicated to the treatment of wounds.

Most of the solutions offered by the pharmaceutical industry are based on synthetic substances, and can produce various side effects. For example, silver sulfadiazine cream, the current topical gold-standard drug in the treatment of burns, delays wound healing, decreases the rate of epithelialization, and in prolonged treatment can lead to the formation of hypertrophic or atrophic scars, can present local cytototoxicity (on keratinocytes and fibroblasts) and can cause systemic disorders such as neutropenia, erythema multiforme, renal toxicity and methemoglobinemia.[1,2]

An alternative to synthetic products is offered by the medicinal plants. They have a long use in human history for the treatment of various skin conditions, including wounds and burns. Numerous current scientific studies demonstrate the ability to heal wounds with herbal preparations,[3-8] and highlight the many benefits, such as low cost, efficacy, ease of application, safety and relatively low side effects (especially local hypersensitivity).

At the same time, the involvement of traditional medicines in multiple mechanisms of wound repair and tissue healing requires evaluation and validation through scientific studies. Among plant bioactive compounds, polyphenols represent a significant class. Gallic acid has antimicrobial, antifungal and antioxidant activity,[9,10] and caffeic acid and its esters have anti-inflammatory, antioxidant action,[11] accelerate the healing of skin wounds,[2] also stimulate the collagen synthesis.[3] Ferulic acid also promotes skin healing by accelerating epithelialization,[12,13] and acts as an antioxidant. Antioxidant activity is a common feature of flavonoids; the protective action of quercetin and rutin on dermal neurovascular cells subjected to oxidative stress has been demonstrated.[14] The presence of rutin in dermatological formulations stimulated wound healing by slowing lipid peroxidation, increasing catalase activity and decreasing protein carboxylation.[15]

## MATERIAL AND METHODS

The objective of this work is an experimental research 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.

The complex formula is based on an extract in olive oil of a mixture of nine medicinal plants, together with other natural ingredients with known wound healing activity, such as:

- Sea buckthorn oil (*Hippophae oleum*)
- Lavender essential oil (*Lavandulae aetheroleum*)
- Viscosity enhancers: coconut oil(*Cocos oleum),* beeswax*(Cera flava)* and conifer resin *(Resina pini).*

The plants used to make the extract were:

1. *Calendula officinalis L., Asteraceae* (marigold)
2. *Matricaria chamomilla L.,Asteraceae* (chamomille)
3. *Symphytum officinale L., Boraginaceae* (comfrey)
4. *Hypericum perforatum L., Hypericaceae* (St. John's wort)
5. *Achillea millefolium L., Asteraceae* (common yarrow)
6. *Arctium lappa L., Asteraceae* (burdock)
7. *Plantago major L., Plantaginaceae* (greater plantain)
8. *Althaea officinalis L, Malvaceae* (marshmallow)
9. *Quercus robur L.,Fabaceae* (oak bark).

The active ingredient is the oily extract of the plant mixture, and the conditioned form is represented by an ointment.

It was expected that experimental data would be obtained to allow the preclinical pharmacological evaluation of the product, tested both as an extract and as a conditioned product (ointment), in order to establish the basic aspects regarding the safety and efficacy of the product.

In order to estimate activity, the following approach using in vitro and in vivo experimental models was considered

**Product safety tests**

1. Culture of reconstituted dermis, in vitro experimental model, for assessing irritant or corrosive activity on the epidermis
2. In vitro pyrogenicity test: testing for the presence of bacterial endotoxin (LAL)
3. Microbial load test

**Efficacy tests**

- Testing of sensitizing potential (in vivo)
- In vitro wound repair test
- Healing of thermal wounds (in vivo)

The testing of this product was performed for the classification of non-invasive medical devices that come into contact with damaged skin, class IIb, in accordance with the medical device regulatory framework under the Council Directive 93/42/EEC of 1993, Annex IX.

The work was done under the **Research project** 108CI from 25.07.2017 (PN-III-P2-2.1-CI-2017-0487) contracted and financed through the Executive Agency for Higher Education, Research, Development and Innovation Funding, Bucharest, Romania.

**Test methodology**

**1. Evaluation of dermal toxicity (Testing irritant-corrosive action on a reconstituted dermis model)**

**Test protocol**

We worked according to the testing protocol provided by the kit supplier, Mattek from Bratislava, distributor of in vitro kits produced by the American company Mattek. Protocol: In Vitro EpiDerm ™ Skin Irritation Test (EPI-200-SIT).

## MATERIAL AND METHODS

Test kit (human reconstituted dermis), delivered in the form of viable cultures, 24 pieces of reconstituted human dermis inserts, in a 24-well plate, inserted in agarose gels and supplemented with culture medium.

Culture medium - DMEM F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) with 10% fetal serum.

Positive control: sodium dodecyl sulfate (SDS), 0.1 g; it is recommended to dissolve it in saline phosphate buffer, for administration, achieving a working concentration of 1%.

Culture plates with 6 wells, culture plates with 24 wells and 96 wells, for the realization of various experimental stages.
"Active Product - Dermaplant Extract "and" Therapeutic Product - Dermaplant Ointment" were tested in three independent replicate series of 3 sets of determinations.

**a) Tissue pre-cultivation**

The tissues are received in the laboratory on the day of arrival (usually the next day of the week).

**Day 1**- After examination of the contents of the package, which must contain the 24-well tissue plate, the culture medium (completely formulated), MTT reagent(3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide), positive control (SDS), phosphate buffered saline (for dissolution of MTT reagent), and solvent for dissolution of formazan resulting from the MTT reduction reaction, culture plates with 6 and 24 wells required for the experiments, the MTT and SDS reagent are stored properly. Each of the tissue inserts is then examined to verify their integrity.

The culture medium is distributed 1 mL in each well of the 6-well plates; 8 such 6-well plates will be prepared and will be preincubated at the $CO_2$ incubator for about 30 minutes, to preheat to room temperature.

Proceed similarly with the plate containing the pieces of reconstituted tissue.

After bringing and stabilizing at 37°C, the plates and tissue are removed from the incubator.
Take each tissue insert, wash in TPS to remove traces of agarose, and place one piece of tissue in three of the 6 wells of a plate. Proceed similarly to all 24 wells.

Incubate overnight (12 hours, 37°C, 5% $CO_2$).

**b) Preparation of test samples**
SDS (positive control) is dissolved in phosphate buffered saline at the concentration recommended in the protocol. The sample does not require weighing, being delivered in the quantity that reconstitutes the concentration applied to the test.

The plant extract samples are administered as an aqueous solution, at a concentration of 10% after a sterilizing filtration, through a 0.2 micron filter. The concentration of 10% exceeds the usual concentrations of topical or skin care products (dermacosmetics). The ointment sample is administered by spatula, spreading evenly 0.2 g on each piece of tissue.

**c) Sample administration and incubation**
**Day 2**
Remove the culture medium from the refrigerator and preheat to 37° C. Remove the necessary 6-well plates from the incubator, which contain the tissue inserts. For all replicas independently, 3 inserts will be used for:
- Positive control
- Negative control
- Samples.

Transfer the pieces of tissue from the upper row of wells to the lower row of wells. Examine again for integrity. Absorb any traces of the environment from the surface of the insert, and wipe with a sterile cotton swab.

Using an automatic pipette, apply exactly a volume of 20 µl of sample (SDS, medium or sample of extract) on the upper surface of the dermis. As some operations will require about 1 minute handling of the parts, the samples will be applied at intervals of 1 minute from each other. The type of sample applied to each of the plates will be marked before administration.

After all the samples have been applied, the pieces of tissue are moved back to the incubator, where they are kept for exactly 35 minutes. After 35 minutes, the pieces of tissue are removed in the hood with laminar flow, where they are kept for another 25 minutes.

**d) Removal of the treated substances and replacement of the medium**
Each of the tissue pieces is washed repeatedly (20 washes with 0.3 mL each) of phosphate buffered saline. Finally, each sample is immersed in TPS, to ensure that the lower face is cleaned of any contaminants. Repeated washing takes about 1 minute, including reinserting the piece. The samples were reinserted into the upper well.

After the treatment of all the pieces is finished, they are reintroduced to the incubator, at 37°C, 5% $CO_2$. They are incubated again for 18 hours under standard conditions.

The MTT reagent is brought to working temperature.

A 24-well plate is marked, in which the MTT reagent will be incubated.

The treated cultures are removed from the incubator. The culture medium is aspirated from each well with pieces; this medium can be stored for testing the secretions of proinflammatory cytokines such as IL-1β and TNFα.

Each of the pieces is carefully wiped off with environmental traces, by dabbing on a sterile filter paper, and placed in a well of the 24-well plate.

The pieces are incubated at 37°C, 5% $CO_2$ for 3 hours. During this time, depending on the existence of viable cells, the MTT reagent will be reduced to an insoluble, blue formazan.

The pieces are extracted from the incubator, and 1 ml of acidified isopropyl alcohol is added over the culture medium. The plate is protected from light and is incubated for 2 hours on a rotary shaker, at room temperature, to solubilize the colored formazan.

Finally, it is allowed to evacuate the entire amount of liquid from the insert. For this purpose, each piece of tissue is perforated with a needle to completely drain the formazan solution.

200 µl of supernatant is taken twice from each well and transferred to a 96-well plate to read the optical density. The optical density is measured in a microplate reader, at a wavelength of 570 nm, compared to a blank represented by acidified isopropyl alcohol. For each of the samples, the average of the 2 readings is calculated. Proceed in a similar way for the other 2 independent replicates.

For each of the samples, % viability is calculated, using the formula:

Relative viability of TS (%) = [$OD_{TS}$ / Average $OD_{NC}$] x 100

Relative viability of NC (%) = [$OD_{NC}$ / Average $OD_{NC}$] x 100

Relative viability of PC (%) = [$OD_{PC}$ / Average $OD_{NC}$] x 100

Where
TS = test substance
$OD_{TS}$ = optical density of the test substance
$OD_{NC}$ = optical density of the negative control
$OD_{PC}$ = optical density of the positive control

For each type of test substance, positive or negative control sample, the average values of relative viability for three individual tissues are calculated and used for classification according to the Predictive Model.

Data interpretation procedure (Predictive model)

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

| In vitro result | Prediction in vivo |
|---|---|
| Average tissue viability <= 50% | Irritant (I), (R38 or GHS category 2) |
| Average tissue viability> 50% | Non-irritant (NI) |

**2. Evaluation of endotoxin loading - LAL method - gel-clot technique**

The bacterial endotoxin test (BET) is used to detect endotoxins in gram-negative bacteria using amoebocyte lysate from Limulus polyphemus or Tachypleus tridentatus. The technique used in the microbiology laboratory for the detection of bacterial endotoxins is LONZA "gel-clot" LAL. The gel-clot method is based on the opacification and gelling reaction of LAL extract in the presence of bacterial endotoxins. Control of bacterial endotoxins (LAL test - gel-clot method) provides an in vitro alternative to the pyrogenicity test performed on rabbits.

**The LAL reagent** used in the test represents a lyophilized product obtained from the lysate of amoebocytes from Limulus polyphemus. The LAL reagent is reconstituted extemporaneously with LAL water, by light and gentle rotations;

**The LAL water** is used for reconstitution of Control Standard Endotoxin (CSE).

Reference Standard Endotoxin (RSE) is a reference preparation with predetermined endotoxin content, specified in the Certificate of Analysis. In case of using RSE endotoxin, before starting the test, the certificate on the manufacturer's website must be accessed and verified, for the expiration date, as it is not mentioned in the certificate of analysis;

Control Standard Endotoxin (CSE) is a reference standard endotoxin (RSE) substitute and has endotoxin content expressed in EU / ng. The potential of bacterial endotoxins to induce a pyrogenic effect is expressed in EU / ng (ml). The potential of the CSE is determined by the ratio between RSE/CSE tested at a certain sensitivity of the LAL reagent.

Reference Standard Endotoxin (RSE) and Control Standard Endotoxin (CSE) are reconstituted with LAL water, according to the instructions specified by the manufacturer in the certificate. In routine testing RSE or CSE is used; after reconstitution the standard series is valid for a determined period of time if kept at 2-8 ºC.

The pH buffer is used only when the pH value of the test solution is outside the normal admissibility values between 6-8. It is used according to the manufacturer's specification and must be free of detectable endotoxins and interference factors.

**Confirmation of LAL reagent sensitivity**

Reference Standard Endotoxin (RSE) or Control Standard Endotoxin (CSE) is a reference preparation with predetermined endotoxin content and which is reconstituted with an amount of LAL water specified in the working procedure specified by the LAL kit manufacturer. The solution resulting from the reconstitution of endotoxin with LAL water is the *stock solution* that has a determined endotoxin concentration and is expressed in IU / ml or EU / ml. Serial dilutions are made so that from the predetermined endotoxin content of the stock solution to reach concentrations of 1 EU / ml, respectively 2λ, λ, λ/2, λ/4 where λ = sensitivity of the LAL reagent used.

The confirmation of the sensitivity of LAL reagent and standard series (RSE / CSE)

- Is made in at least 4 replicates;
- Is performed before the start of the test cycle;
- Is performed when a new stock solution is reconstituted;
- Is performed when using a new batch of reagent.

Each replicate consists of 0.1 ml of LAL reagent, reconstituted extemporaneously according to the working procedure specified by the kit manufacturer, added over 0.1 ml of the concentrations 2λ, λ, λ/2, λ/4 of RSE/ CSE.

The mixture will be incubated according to the manufacturer's specification at 37 ± 1 ° C for 60 + 2 min.

The result of the standard series is not valid if:

- One of the replicates at the lowest concentration of the standard series λ/4 induces a positive result

represented by a firm gel which remains stable when the tube is turned at 180°;
- One of the replicates at the 2 X concentration induces a negative result.

**The testing of endotoxin limit (L)** for a sample involves
- Sample dilution (if necessary)
- Positive control standard series (C +)
- Negative control (C-)
- Positive sample control (PSC)

The endotoxin concentration in the product to be analyzed must be lower than the permitted endotoxin limit. To test the endotoxin limit it is necessary to prepare dilutions by dissolving or diluting the active substances or medicinal products in LAL water.

**Control tests**
- Negative control test (C-) consisting of 0.1 ml of LAL water over which 0.1 ml of LAL reagent is added;
- Positive control test (C +) consisting of 0.1 ml CSE dilution at twice the concentration, over which 0.1 ml LAL reagent is added;
- The positive sample control (PSC) test is performed by combining 0.05 ml of PSC sample dilution, to which 0.05 ml of endotoxin is added, with 0.1 ml of LAL reagent;
- Testing the sample consists in adding 0.1 ml LAL reagent over 0.1 ml of the sample dilution.

The distribution of the reagent in all test tubes was done in maximum 2 minutes. After distribution of the reagent, the test tubes were gently shaken to homogenize the contents, and then the test tube stand was incubated in a thermostated bath at 37° C ± 1° C for 60 + 2 min. After the expiration of the incubation period, the results are read.

***Conformity of the result***
**The test result is consistent if**
In both replicates with **the sample (or dilution) to be analyzed**, the result is **negative** (the concentration of endotoxin in the sample analyzed is below the maximum allowed level), i.e. the reaction product is not a gel or a viscous substance is formed which is not maintained in inside the tube after rotating it 180°.

The product is considered as **compliant** if a **negative** result is found in both replicates of the sample to be analyzed.

The product is considered **non-compliant**, if in both replicates the result is **positive** (the endotoxin concentration in the product to be analyzed is greater than or equal to the maximum allowed limit).

**3. Test method for microbial contamination**
Equipment and glassware: Petri dishes with a diameter of 90 mm, sterile, disposable; sterile graduated pipettes of volume 10 ml; sterile test tubes; sterile Erlenmayer glasses; automatic pipette; analytical balance; biological cabinet with laminar air flow (LAF); thermostats set at 25, 35 and 44° C.

Culture media used (as per European Pharmacopoeia):
- Medium A (Casein soya bean digest broth)
- Medium B (Casein soy bean digest agar)
- Medium C (Sabouraud-glucose agar with chloramphenicol)
- Buffer solution peptoned water with sodium chloride, pH = 7
- Medium E (Enterobacteria enrichment broth-Mossel)
- Medium D (Lactose monohydrate broth)
- Medium F (Crystal violet, neutral red, bile agar with glucose)
- Medium N (Cetrimid agar)
- Medium O (Baird-Parker agar)
- Medium I (Tetrationat bile brilliant green broth)
- Medium K (XLD agar).

Test methodology total number of viable aerobic microorganisms - inoculation method in depth.

The samples are processed under aseptic conditions, in the class A working point. The dilution 1/10 is prepared: 10 g or 10 ml of product are dissolved in 90 ml of buffer solution and homogenized. Subsequently, serial dilutions (1/100, 1/1000) are prepared using the same solvent. 1 ml of the sample dilution is distributed in 2 sterile Petri dishes and 15-20 ml of culture medium B, melted and cooled to a temperature of 45° C are added to each plate.

Each Petri dish is covered with the lid, rotated gently on a flat surface and allowed to solidify at room temperature. The plates are thermostated with culture medium B at a temperature of 30-35° C for 5 days.

**RESULTS**

After the expiration of the incubation period, Petri dishes with microbial growth are selected, but microbial colonies are counted only from plates with a maximum of 250 colony forming units (cfu). The total number of viable aerobic microorganisms is equal to the arithmetic mean of the number of cfu recovered from culture medium B. If no microbial recovery is obtained, the result is expressed as less than the limit of detection (ie the lowest dilution tested) namely <10 cfu/g or ml.

**Test methodology total number of yeasts and molds - in-depth inoculation method**
The samples are processed in the same way as for the total number of viable aerobic microorganisms: serial dilutions (1/10, 1/100) are prepared using buffer solution as solvent and 1 ml of the sample dilution is distributed in 2 sterile Petri dishes. 15-20 ml of culture medium C (Sabouraud glucose agar with chloramphenicol)is added to each plate, melted and cooled to a temperature of 45° C.

Each Petri dish is covered with the lid, rotated gently on a flat surface and allowed to solidify at room temperature. The plates are thermostated with culture medium C at a temperature of 20-25° C for 5 days.

**Results**
After the expiration of the incubation period, Petri dishes with microbial growth are selected, but microbial colonies are counted only on plates with a maximum of 50 cfu. The total number of yeasts and molds is equal to the arithmetic mean of the number of cfu recovered from culture medium C. If no microbial recovery is obtained, the result is expressed as less than the detection limit (ie the lowest dilution tested) namely <10 cfu/g or ml.

Test methodology for **Enterobacteria and other Gram negative bacteria**
In 100 ml of culture medium D add 10 g / ml product (1/10 dilution), homogenize and incubate at 20-25 ° C for 2h. After incubation, 1 ml (from 1/10 dilution) is transferred to 100 ml of culture medium E which is thermostated at 30-35° C for 18-48 h. Subcultures are performed by inoculating on the surface two plates of culture medium N (1 ml in each plate) and incubated at 30-35° C for 18-72 h.

**Result**
After the expiration of the incubation period, the Petri dishes with microbial growth are selected. The appearance on the culture medium F of some red bacterial colonies, with a red area around it can be the indication of the presence of Enterobacteria.

**Test methodology for Pseudomonas aeruginosa**
10 ml (equivalent to 1 g / ml product) of the 1:10 dilution of the sample prepared as a total number of viable aerobic microorganisms are added to 100 ml of culture medium A, mixed and incubated at 30-35° C for 18-48 h. Subcultures are performed by inoculating two plates of culture medium N on the surface (1 ml in each plate) and incubating at 30-35° C for 18-72 h.

**Result**
After the expiration of the incubation period, Petri dishes with microbial growth are selected. The appearance on the culture medium N of some yellow-green bacterial colonies can be the indication of the presence of P. aeruginosa.

**Test methodology for Staphylococcus aureus**
10 ml (equivalent to 1 g / ml product) of the 1:10 dilution of the sample prepared as a total number of viable aerobic microorganisms are added to 100 ml of culture medium A, mixed and incubated at 30-35° C for 18 -48 h. Subcultures are then performed by inoculating two plates of culture medium O on the surface (1 ml each in each plate) and incubating at 30-35° C for 18-72 h.

**Result**
After the expiration of the incubation period, Petri dishes with microbial growth are selected. The appearance on culture medium O of yellow or white bacterial colonies and surrounded by a yellow area can be the indicator of the presence of S. aureus.

**Test methodology for pathogenic microorganisms from the genus Salmonella**
10 g / ml of the sample are added in 100 ml of culture medium A, homogenized and incubated at 30-35° C for 18-48 h. After incubation, 0.1 ml (from 1/10 dilution) is transferred to 10 ml of medium of culture I which is thermostated at 43-45° C for 18-48 h. Subcultures are then performed by inoculating two plates of culture medium K on the surface (1 ml each in each plate) and incubating at 30- 35° C for 18-72 h.

**Result**
After the expiration of the incubation period, Petri dishes with microbial growth are selected. The appearance on the culture medium K of some reddish bacterial colonies can be the indicator of the presence of Salmonella sp.

**Test methodology for Escherichia coli**
10 ml (equivalent to 1 g / ml product) of the 1:10 dilution of the sample prepared as a total number of viable aerobic microorganisms are added to 100 ml of culture medium A, mixed and incubated at 30-35° C for 18-24 h. After incubation, 1 ml is transferred to 100 ml of culture medium G which is thermostated at 43-45° C for 24-48 h. Subcultures are performed by inoculating 1 ml each on the surface of two plates of culture H and incubating at 30-35° C for 18-72 h.

**Result**
After the expiration of the incubation period, Petri dishes with microbial growth are selected. The appearance on the culture medium H of some bacterial colonies can be the indicator of the presence of E. coli.

**4. Determining the cells viability**
We used 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.
In the case of the ointment, the solubilization was done in DMSO (1:10), followed by dilution of the solution in the working medium.

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.

**5. Tests for repairing thermal burns**
**The mouse model**
The model uses young mice (6-8 weeks old) that are anesthetized with ketamine and xylazine or other

anesthetics given intraperitoneally. In some cases, 1 ml of saline is administered subcutaneously along the spine to protect the spinal cord from any injury. Subsequently, the hair on the back is removed. The dorsal region is the area of choice, because the animal does not have access and there are no additional scratches in the area of the thermal injury. The mouse is then fixed with the epilated region in a template with a window that ensures a predetermined exposure of the skin. The exposed surface of the mouse in the template is then immersed in a water bath at 60-100° C for 8-12 seconds to cause a total burn. The animals are then frequently seen for signs of pain or discomfort and given painkillers.

In mice, the thermal injury is limited by the size of the animals, as they can only tolerate a burn of 30% of the total body surface. Some authors mention that the hypermetabolism phase is only fully activated by thermal damage greater than 40% of the total body surface. Thus, although the mouse burn model is simple to perform, it loses relevance when studying the hypermetabolic status after burn injury.

**The rat model**

The rat burn model is performed in the same way as the mouse model, with some minor differences, such as temperature and duration of exposure to heated water. Rats, being larger animals, can withstand a burn of up to 60% of the total body surface, by using the aforementioned model for the dorsal region and additionally another thermal injury in the abdominal region. The creation of a thermal burn wound with over 60% of the total body surface in rats is followed by a low degree of survival and is not sustainable for experimentation. There is a need for a sufficiently extensive thermal injury model to cause the hypermetabolism observed in large-scale human burns. Because in the initial post-burn stages in humans, glucose is extracted from the affected tissue, it induces hyperglycemia and hyperlactatemia.

Therefore, the rat burn model is superior to the mouse model by the increased ability to recover hypermetabolism, it becomes deficient when trying to include the risk of infection to achieve post-burn sepsis observed in human patients with extensive burns over 60%.

**6. Determining the level of cytokines**

An extension of the research was proposed, by applying a model to evaluate the level of cytokines, chemokines and lymphokines pro- and anti-inflammatory in the serum of treated animals. This work was carried out outside the initial research project, as the level of funding and the duration of the contract would not have allowed these determinations to be made.

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 (Fig. 1). The treatment consisted of the daily application of the ointment preparation, for 14 days (Fig. 2, 3). No occlusive dressing was used. The animals were fed and received water ad libitum. No adverse effects were reported, although recovery of the control group was slower.

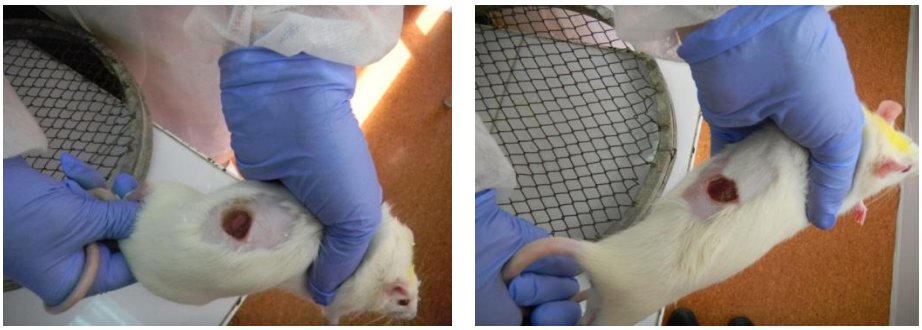

**Fig. 1: The wounds in the first day, in the treated (left) and non-treated (right) groups.**

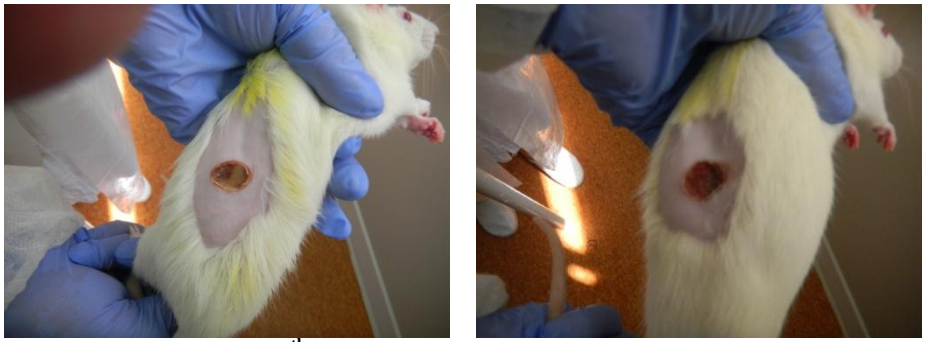

**Fig. 2: The wounds in the 7$^{th}$ day, in the treated (left) and non-treated (right) groups.**


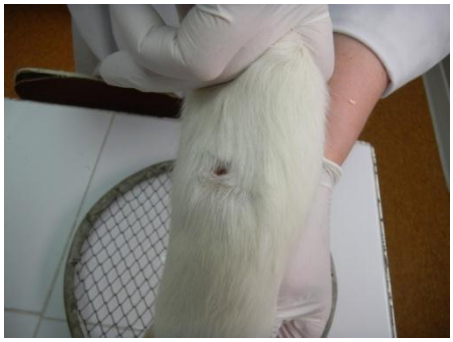

**Fig. 3: The wounds in the 14th day, in the treated (left) and non-treated (right) groups.**

Cytokine levels - IL1β, TNFα and IL6 were determined in the culture supernatants collected according to ToxDerm kit experiment, using commercial ELISA kits, in triplicate.

## RESULTS AND DISCUSSION

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

Viability tests were performed, by applying the EPI-SKIN model, on the products "Active product - Dermaplant extract" and "Therapeutic product - Dermaplant ointment" in three series of independent replicates, of 3 sets of determinations in each independent replicate.

**The data recorded after performing the test were**

| Product | Media 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% |

* Average of three independent replicates
** Positive witness; 1: negative witness; 2: student t test, 2 queues, homoscedactic distribution, towards positive control

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 in accordance with the instructions use.

### 2. Evaluation of endotoxin loading

The endotoxin load was estimated by the LAL test for the Dermaplant Extract preparation, due to the fact that the product in the form of an ointment can possibly be contaminated with endotoxins coming from the plants used in the manufacturing process. The ointment has characteristics that make it impossible to apply the endotoxin test.

According to the obtained data, the product Dermaplant - Extract was found to be within the recommended endotoxin content limits for topical products and type II medical devices, respectively below 0.5 EU / mL.

### 3. Evaluation of microbial contamination

At the evaluation, Dermaplant Extract and Dermaplant ointment product were used for testing, in the conditions of determining the bacterial, fungal and yeast contaminants, according to the described protocol.

Following the evaluation, no contaminants were found in the category of pathogenic microorganisms.

At the same time, although it has a microbial load represented by non-pathogenic species, the product in its current form is not yet recommended for medical devices that come into contact with damaged tissues, for which a new sterilization operation is recommended, most likely by irradiation, which would ensure the recommended level of sterility for burn treatment products.

### 4. Determining the cells viability

We used mouse embryonic cells cultures 3T3 - NIH/3T3 (ATCC CRL-1658), according to the above mentioned protocol.

Dermaplant Extract demonstrated a remarkable proliferation-stimulating capacity (+ 18% increase in proliferation rate).

### 5. Testing the ability to promote the repair of burn wounds

The test was performed using the rat model. We worked on white Wistar rats, males, of 180-200 g, provided by the biobase of the Cantacuzino institute. Two variants of

the test model were applied, namely the conventional model, in which the lesion was performed on both sides of the animal, and a modified model, in which the thermal lesion was performed on one side, and a lot of "control" animals was used (in which the lesion is not treated) and a "treated" lot in which the tested preparation was administered.

In both cases, the wounds were obtained by applying an exposure of the epilated dorsal skin at a temperature of 90°C for 10 seconds on animals anesthetized with ketamine (50 mg / kg, intraperitoneally). Following this exposure, the animals were allowed to rest for 24 hours, after which the treatment was applied.

**The lots were formed**

| Lot No. | Lot name | Obs. |
|---|---|---|
| 1 | Treated lot | Bilateral thermal injury |
| 2 | Control lot | Unilateral thermal injury |
| 3 | Treated lot 2 | Unilateral thermal injury |

The lots were treated every 48 hours for 6 days.

The results of the experiments - visual observations of tissue regeneration

| Lot No. | Lot name | Obs. | Result (% lesion area) |
|---|---|---|---|
| 1 | Treated lot | Bilateral thermal injury - control area | 52% |
| | | Bilateral thermal injury - treated area | 41% |
| 2 | Control lot | Unilateral thermal injury | 31% |
| 3 | Treated lot 2 | Unilateral thermal injury | 56% |

From the analysis of the results, it can be deduced that in the case of bilaterally injured animals, there is probably an influence (probably mediated by the production of cytokines and lymphokines) that generates an improved repair on the untreated flank.

On the "unilateral" model, a clearer stimulation of the repair process and a more advanced re-epithelization were observed in the treated animals, compared to the animals in the control group.

**6. Determining the level of cytokines**

In order to deepen these aspects, we continued the research, by using multiplex analysis kits to monitor the effect of the product on the production of cytokines and lymphokines involved in the inflammatory process and then in wound repair.

Significant reductions in the level of proinflammatory cytokines IL1β and TNFα were recorded, as well as a maintenance level comparable to the control for IL6 (Fig. 4).

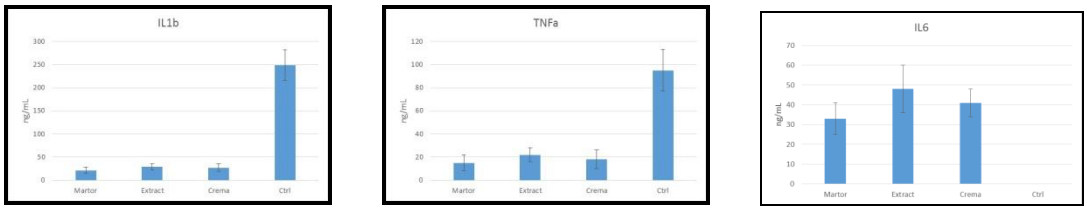


**Fig. 4: The level of cytokines: IL1β, TNFα and IL6, compared to control.**

## CONCLUSIONS AND PROPOSALS

The works performed on the products Active product - Dermaplant extract and Therapeutic product - Dermaplant ointment have demonstrated the following:

- The preparations do not show dermal toxicity, as demonstrated by the use of In Vitro EpiDerm ™ Skin Irritation Test (EPI-200-SIT)
- Safety tests have shown that the product is free of endotoxin-like contaminants and is therefore safe for application on injured skin.
- The presence of pathogenic germs was not detected, and the microbial load was within the limits imposed by the Pharmacopoeia for topical preparations.
- Dermaplant Extract, tested in vitro on 3T3 fibroblasts demonstrated a remarkable ability to stimulate proliferation (+ 18% increase in proliferation rate)
- The evaluation of the "in vivo" repair effect showed a stimulation of the repair of the damaged dermal tissue, on a model of unilateral thermal injury in rats, the repair process at 6 days being accelerated by 69%.
- The evaluation of the level of cytokines, chemokines and lymphokines in the serum of treated animals highlighted significant reductions in the level of proinflammatory cytokines IL1β and TNFα.

The pharmacodynamic and toxicological studies highlighted noteworthy proliferation-stimulating and wound healing activities of the product DERMAPLANT, without toxic, adverse or irritant effects. The ointment proposes a topical composition for burns and wounds, which is effective and well tolerated. The product has also good bioavailability, being fully absorbed in the examination 24 hours after application. Further research

is needed to assess the entire range of ointment effects, indications and modalities of administration.

## REFERENCES


1. Aziy Z, et al - Systematic review of silver-containing dressings and topical silver agents for burn wounds, *Burns*, 2012; 38(3): 307-318.
2. Atiyeh BS, et al - Effect of silver on burn wound infection control and healing: review of the literature, *Burns*, 2007; 33(2): 139-148.
3. Majewska I, et al. - Proangiogenic activity of plant extracts in accelerating wound healing – a new face of old phytomedicines, *ActaBiochim Pol*, 2011; 58(4): 449-360.
4. UpadhyaY NK, et al. - Safety and wound healing efficacy of sea buckthorn (*Hippophaerhamnoides*L.) seed oil in experimental rats, *Food ChemToxicol*, 2009; 47(6): 1146-1153.
5. Deters A, et al – Aqueous extracts and polysaccharides from Marshmallow roots (Althea officinalis L.): cellular internalisation and stimulation of cell physiology of human epithelial cells in vitro, *J Ethnopharmacol*, 2010; 127(1): 62-69.
6. Amini M, et al – Effect of Plantago major on burn wound healing in rat, *J Appl Animal Res*, 2010; 37(1): 53-56.
7. Nevin KG, et al - Effect of topical application of virgin coconut oil on skin components and antioxidant status during dermal wound healing in young rats, *Skin Pharmacol Physiol*, 2010; 23(6): 290-297.
8. Ghelardini C, et al - . Local anaesthetic activity of the essential oil of Lavandula angustifolia, *Planta Med*, 1999; 65(8): 700-703.
9. Preethi KC et al - Wound healing activity of flower extract of Calendula officinalis, *J Basic Clin Physiol Pharmacol*, 2009; 20(1): 73-79.
10. Jarrahi M, et al - Evaluation of topical Matricaria chamomilla extract activity on linear incisional wound healing in albino rats, *Nat Prod Res*, 2010; 24(8): 697-702.
11. Kalita D, et al - Antimicrobial activity of leaf extracts of two medicinal plants of Boghoral Hill (Morigaon) against human pathogens, *The Bioscan*, 2012; 7(2): 271-274.
12. Ghaisas MM, et al - Evaluation of wound healing activity of ferulic acid in diabetic rats, *Int Wound J*, 2014; 11(5): 523-532.
13. Xiyiun Y, et al - inventors; East China Normal University, applicant. Application of ferulic acid in preparation of medicine used for promoting skin wound healing. State Intellectual Property Office of the P.R.C. patent CN, 2012; 102342926A.
14. Skaper SD et al - Quercetin protects cutaneous tissue-associated cell types including sensory neurons from oxidative stress induced by glutathione depletion: cooperative effects of ascorbic acid, *Free Radic. Biol Med.*, 1997; 22(4): 669-678.
15. Almeida JS, et al - Hydrogels containing rutin intended for cutaneous administration: efficacy in wound healing in rats, *Drug Dev Ind Pharm*, 2012; 38(7): 792-799.