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A proprietary Rosemary based terpenic composition for topical scar management post Acne treatment

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1. Introduction

Acne vulgaris is one of the most common skin conditions that affects people of all ethnicities and age-groups. It is caused by inflammation of the pilosebaceous unit and can be triggered by a variety of factors, such as excess oil production, bacteria, hormonal imbalances, and genetics[1,2]. The presumed pathophysiology involves alteration of keratinization within the pilosebaceous unit resulting in the formation of comedones, increased sebum production, proliferation of *Cutibacterium acnes* (*C. acnes*), and production of perifollicular inflammation[3]. The early preclinical inflammation in acne persists throughout the acne lesion's life cycle, from micro comedones to closed comedones to inflammatory lesions and eventually to post inflammatory erythema (PIE), post-inflammatory hyperpigmentation (PIH), and scarring[4].

Acne scars appear most often in people struggling with severe forms of acne. Scar formation is part of the wound healing process, which is divided into three phases: inflammatory phase, healing or proliferative phase, and remodeling phase[5]. The inflammatory phase is marked by the activation of resident immune cells and infiltration of circulating immune cells. The second phase is the proliferative phase wherein the proliferation and migration of fibroblasts, keratinocytes, and stem cells occur, in addition to the secretion of extracellular matrix (ECM) and angiogenesis. Finally, the wound-healing program culminates in the remodeling phase which comprises the removal of excess ECM and restructuring of cell-cell and cell-matrix interactions. Impairment of any of these phases can lead to delays in the kinetics of the wound-healing program[6]. PIE results from wound healing related microvascular dilatation that is perceived as general redness, not visible telangiectasia, which is exacerbated by repair related epidermal thinning[7].

Treatment of acne scarring creates a challenge for both patients and dermatologists. Multiple options are available for scar treatments: laser surgery, radiofrequency intervention, chemical peels, cross technique, dermabrasion, needling, subcision, punch techniques, fat transplantation, and other tissue augmenting agents[8]. Other topical treatments include drugs like polymyxin B,[9,10] Oncostatin M[11] and other platelet-derived growth factor (PDGF) in combination with antibiotics work on stimulating epithelialization, wound contraction and reduction in wound volume. Retinoids[12] and Corticosteroids[13] are also commonly used for scar management as they promote collagen synthesis, re-epithelialization and angiogenesis. These options are, however, either quite intrusive or have strong side-effects like increased skin dryness and redness, stinging sensation and increased sensitivity to the sun[14]. Therefore, there remains a

strong need for more natural solutions to address the wound healing aspect of acne scarring more holistically.

Historically, Aloe vera gel has been reported for treating burns and ulcers and has been shown to reduce pain and improve healing time[15]. Certain polyphenols like Curcumin have shown to modulate all the four phases of wound healing and reported to inhibit the production of TNF-α and IL-1 via NF-κB signaling, which are key cytokines in mediating inflammation[16,17]. Recent studies have also shown the potential of Alginate biopolymer in wound healing due to their biocompatibility and non-toxicity[18]. Though the use of natural actives in wound healing holds significant promise, the mechanism by which they exhibit these properties is not fully understood and even the ideal formulation and delivery method for natural actives to maximize their efficacy and minimize potential adverse effects requires more extensive research[19].

Hydroalcoholic extracts of *Rosmarinus officinalis* (Rosemary) and its essential oil have been reported multiple times in the past for their wound healing ability. These extracts mostly contain compounds like rosmarinic acid, carnosic acid while the essential oil is mostly composed of volatile terpenes and waxes[20,21]. The rosemary leaves are also abundant in triterpenoids but this category of compounds have been largely unexplored despite their strong activity as antimicrobials and anti-inflammatory agents[22]. The triterpenoids like ursolic acid and oleanolic acid have long been known for their anti-inflammatory activity, collagen synthesis, promotion of angiogenesis and wound healing property with improved aesthetic results[23]. We selected nature derived triterpenic acids that bear terpenoidal scaffold close to pharmaceutical references such as dexamethasone which are used as anti-inflammatory and wound healing drugs, making them suitable molecules of interest to design the extraction process around. Additionally, despite strong proven efficacy of ursolic acid and oleanolic acid, their skin absorption potential is quite low, limiting their use in topical skin applications[24].

The present work highlights our novel and unique extract coming from the leaves of *Rosmarinus officinalis*, designed to contain more than 70% of triterpenic acids including ursolic, oleanolic, betulinic and micromeric acid which was further salified to improve its bioavailability and to have a more drug-like performance. Our proprietary extract shows promising results in wound closure activity in an in-vitro reconstructed skin model.

2. Materials and Methods

Novel Rosemary extract

The leaves of the *Rosmarinus officinalis* plant were powdered using the IKA® Pilotina dry-milling system and sieved through 100-µm mesh to afford a coarse powder. The resulting powder (100 g) was extracted using 100% Ethyl Acetate (1:10 m/v) for three consecutive cycles at 70 ºC at 300 rpm. After three cycles of extraction, the mixture was filtered and the combined filtrates were reduced under pressure using Rotary evaporator and concentrated to afford a syrupy mass. To this, 100 ml of cold Ethyl acetate was added which yielded a white precipitate. The white precipitate was then filtered out using Whatman No.1 Filter paper. After three crops of precipitation, the precipitates were combined and washed with cold ethyl acetate and filtered again to remove traces of green chlorophyll. The process afforded 5-6g of white colored solid precipitate containing 38-40% Ursolic acid, 15-20 % Oleanolic acid, 3-4% of Betulinic acid and 8 -10% of Micromeric acid. This Rosemary extract was further treated with KOH to obtain a salified extract of triterpenic acids.

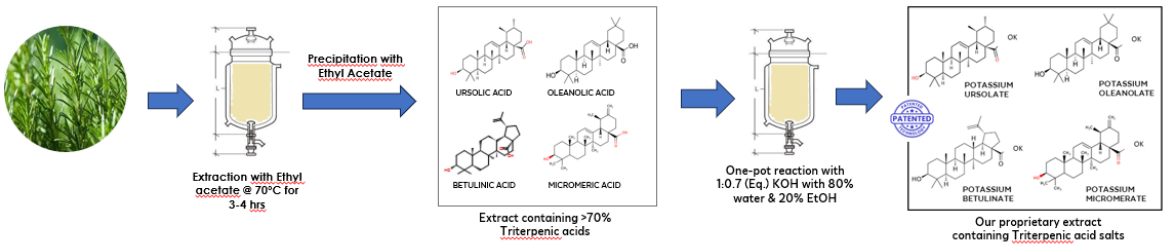


Figure 1: Patented process for obtaining our novel Rosemary extract

Wound Healing Evaluation

The wound healing efficacy of the Rosemary extract was evaluated on the re-epithelialization migration TSkin™ model at Episkin, Lyon, France. This migration model mimics phase III and IV of wound healing process. The goal of the migration test is to observe the capacity of actives to improve the skin regeneration through the epithelialization step of wound healing. The T-Skin™ lattice was ballasted by a metal ring (Ø1cm) to create a modified version called Migration T-Skin™ model[25]. Keratinocytes were seeded on the living dermal equivalent around the ring while the center of the ring remaining empty. After 6 days of immersion in culture medium, the tissue samples were lifted to the air-liquid interface inducing epidermal differentiation and stratification. Finally, the ring was removed after D10 and stored at 37°C until the end of the study at D20. Oncostatin M (OSM) and Vitamin C (VITC) were used as pro-epithelialization controls. The test samples or controls were added to the culture medium at Day 10 with the treatments being renewed at regular intervals. At the end of treatment (D20), Migration T-Skin™ model were fixed in 4% neutral buffered formol, dehydrated in graded alcohol/isopropanol and embedded in paraffin blocks.

In this migration study, to assess tissue features and to make quantitative measurements objectively for follow 3D closure kinetics, EPISKIN developed a faster and robust method based on a non-invasive Optical Coherence Tomography (OCT) approach combined with a final quantification of the histological quality. OCT is a technique based on the analysis of infrared light reflected by tissues and on the creation of an interference signal[26]. OCT is capable to image thin sections within thick, living biological tissues nondestructively and without exogenous dyes by measuring their optical reflections. Through optical acquisition and processing with specifics algorithms combined with a final quantification, epidermal and dermal thicknesses, and epidermis profile of the tissue after reconstruction are also determined. Then, global morphology of the treated tissues is scored at the end of treatment by histology[27].

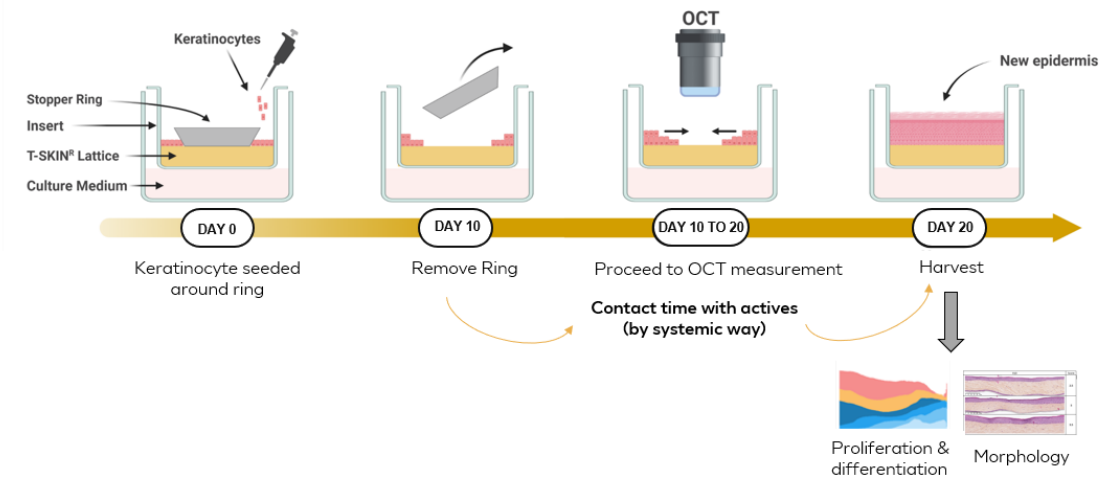
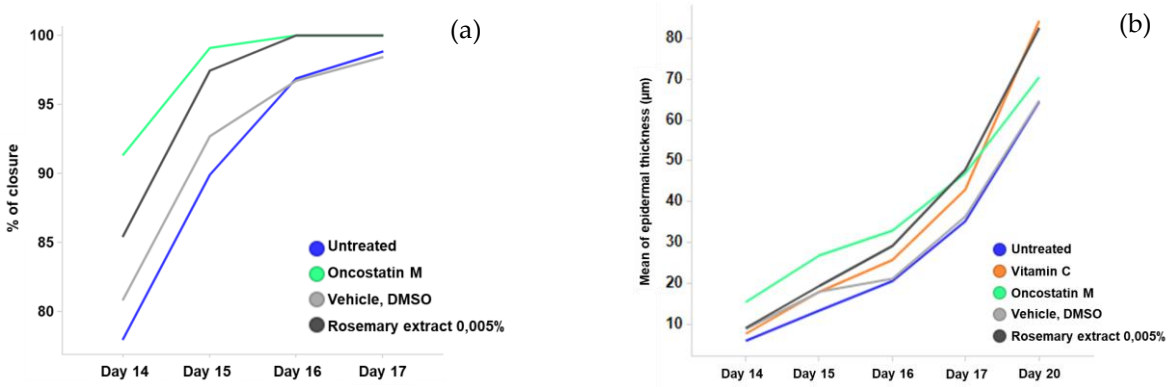


Figure 2: Methodology for Evaluation - The migration model mimics the phase III and IV of wound healing process

3. Results

The two clinical references used in the present evaluation showed positive effect on the test. Oncostatin M (OSM) showed an increase in the speed of migration and proliferation of keratinocytes (equivalent to wound closure) while Vitamin C improved the quality of reconstruction, organization and differentiation (equivalent to quality of re-epithelialized skin). The patented rosemary extract was tested at the highest non-cytotoxic doses in triplicates in this evaluation. The extract at 0.005% showed increase in the kinetics of wound closure at Day 15 & Day 16 which was similar to the control, Oncostatin M (Graph 1 (a)). The extract at 0.005% also demonstrated through the epidermal thickness, proliferative effect at the earliest maturity stage (between both positive references) and differentiation effect at day 20 which was similar to the effect demonstrated by Vit C (Graph 1 (b)).



Graph 1 : (a) Closure speed, kinetics of closure were determined by image analysis using the newly developed software (untreated TNT vs Oncostatin M OSM as positive controls, n=9.

b) Epidermal thickness, mean of tissues (n=9) determined by image analysis using the newly developed software (untreated TNT vs Vitamin C VITC and Oncostatin M OSM as positive controls).

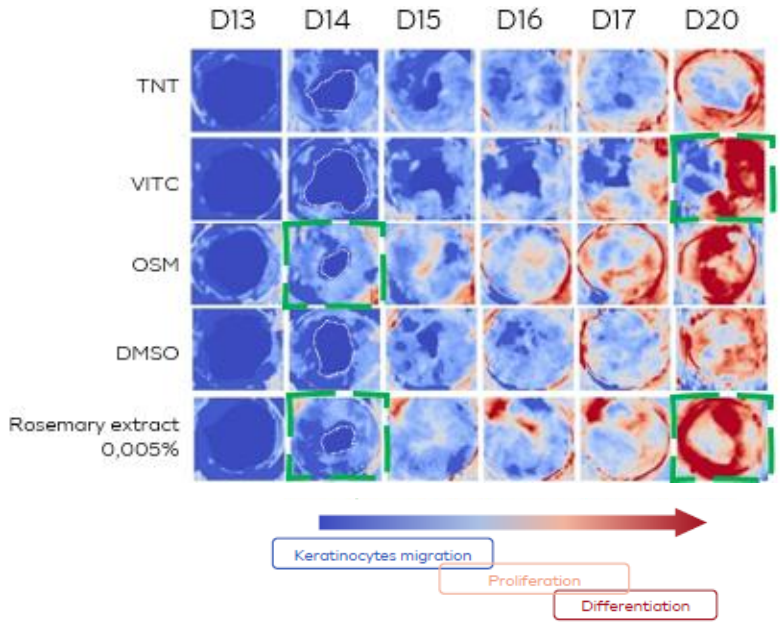


Figure 3: OCT Visualizations : example of epidermal thickness profile images on one sample by raw materials (gradient thickness from blue (thinner) to red (thicker))

The Figure 3 shows the OCT visualization images of the wound contraction along with the epidermal thickening gradient on day 13 to day 17 and day 20 for the untreated control (TNT), Vitamin C (positive reference), Oncostatin M (OSM) (Pharma reference), DMSO (vehicle) and the Rosemary extract at 0.005%. The figure shows the diminishing wound size in all the treatments from Day 13 to Day 20. As can be seen from the figure and also the table 1 below, OSM shows a much higher rate of wound contraction at Day 14 (91%) and Day 16 (100%) versus the positive control Vit C (76% and 91%) and the untreated control (78% and 96%). The rosemary extract shows 85% closure on day 14 and 100% closure on Day 16. The example of OCT images in figure 3 (representing all the treatments) also show the migration of keratinocytes on day 14 visible as light blue clouding around the wound.

4. Discussion

The rosemary extracts have previously been reported by Basal et al to have wound closure activity in the alloxan-induced-diabetic model.[21] The aqueous extract showed wound closure of 88% versus the untreated control at Day 15 at a dosage of 0.2 ml injected through IP (10% dilution in water v/v). On the contrary, the essential oil of rosemary showed a closure of 92% in the same experiment when applied topically at a dosage on 25 **µL**. The wound healing activity of Rosemary oils and its aqueous and hydroalcoholic extracts has been mostly attributed to its antimicrobial, anti-inflammatory, antioxidant effects and collagen synthesis effects.[28,29] The main constituents responsible for these activities are monoterpenes and oxygenated monoterpenes like pinenes and 1,8-cineole which comprise almost 60% of the rosemary essential oil.[30] Diterpenes like Carnosic acid and carnosol are the key anti-inflammatory agents while phenolic acids like Rosmarinic acid are the key antioxidants in the hydroalcoholic extracts of rosemary.[28]

The aerial parts of rosemary are also rich in triterpenoids like ursolic acid, oleanolic acid, betulinic acid and micromeric acid which have also been known for their healing properties of topical wounds apart from strong antimicrobial and anti-inflammatory activity. Despite the huge potential, this class of compound has been largely ignored in the plant and most commercial extracts of rosemary focus only on rosmarinic acid and the essential oils. A study by Al-Musawi et al have shown the wound healing potential of ursolic acid and κ-Carrageenan (KG) containing wound dressings. The ursolic acid and KG were electrospun in the nanofiber mats composed of polyvinylpyrrolidone (PVP).[31] The nanofibers containing 0.25 % ursolic acid exhibited the highest porosity, hydrophilicity, and degradation rate and a wound closure rate of 60 %, 2.5 times higher than that of the control group in a diabetic rat model. Multiple reports on the healing mechanism of ursolic acid suggest that the activity of this molecule could be attributed to stimulation of keratinocyte proliferation, collagen synthesis and extracellular matrix remodeling, anti-inflammatory and antimicrobial effects along with upregulation of epidermal growth factors.[32,33] Oleanolic acid has also been reported to cause the activation of epidermal growth factor resulting in the enhancement of cell migration. The compound also modulates the ECM organization by modulating the type I and type III collagen resulting in the increased flexibility and reduction of hypertrophic scar formation[34,35].

Earlier our patented rosemary extract comprises of these triterpenic acids, namely ursolic acid, oleanolic acid, betulinic acid and micromeric acid, which were further salified with potassium hydroxide to yield the salts of these acids to enhance their aqueous solubility. The efficacy of this salified extract in wound healing is being reported for the first time in a human reconstructed skin model.

The OCT visualization images in Figure 3 show the kinetics of wound contraction from Day 13 to Day 20 for the untreated control, the positive control (Vitamin C), the pharmaceutical reference (OSM) and the rosemary extract. The table 1 below summarizes the effect of the extract on the wound closure speed versus Vitamin C, OSM and the untreated control. The extract at 0.005% demonstrated wound closure of 85% at Day 14 and 100% at Day 16 which was similar to the control, Oncostatin M (91% at Day 14 and 100% at Day 16). The highest rate of contraction can be seen with OSM and the rosemary extract at 0.005%. The figure 3 also shows the migration of keratinocytes, their proliferation and differentiation as a gradient from light blue to red. As can be seen from the figure, the highest rate of keratiocyte migration (light blue coloration) is demonstarted by OSM and the rosemary extract at 0.0005% on Day 14 versus the Vit C and untreated samples. Day 20 shows the keratinocytes maturation and the resulting epidermal thickening for all the treatments visible as red coloration in the figure 3. The figure indicates that vitamin C shows the highest amount of epidermal thickening followed by OSM and the rosemary extract. The results clearly indicate that the wound healing potential of rosemary extract is comparable to the pharma reference OSM in terms of wound closure percentage and also the epidermal thickness. To the best of our knowledge, this is the first report on the wound healing activity of a salified triterpenoid based rosemary extract in a reconstructed skin model with performance close to the pharmaceutical reference.

*Table1: Mean Closure Speed percentage of Untreated control, Vitamin C, Oncostatin M and Rosmarinus officinalis extract at 0.005% on Day 14, 15 and 16 (**pvalue<0.01; ***pvalue<0.001)*

MEAN VALUE 24MIG07: 9 samples	CLOSURE SPEED (%)		
	D14	D15	D16
UNTREATED CONTROL	78,0 ± 7,0	89,9 ± 4,8	96,9 ± 2,8
VITAMIN C	76,1 ± 4,6	87,5 ± 3,9	91,3 ± 2,8 ***
ONCOSTATIN M	91,3 ± 3,9 ***	99,1 ± 1,0 ***	100 ± 3,0 **
ROSEMARINUS OFFICINALIS-0,005%	85,4 ± 9,9 **	97,5 ± 3,5 ***	100 ± 3,0 **

5. Conclusion

The current evaluation shows that this proprietary Rosemary extract demonstrates wound healing activity similar to the clinical references used and has a great potential for application in treatment of post acne scars and marks.

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