

PROPOLIS - AN ANCIENT REMEDY OR A NEW PARADIGM FOR WOUND HEALING: IN-VIVO PRECLINICAL EVALUATION, ANTIMICROBIAL ACTIVITY AND HISTOPATHOLOGIC ASPECTS

PROPOLISUL - UN REMEDIU ANCESTRAL SAU O NOUA PARADIGMĂ ÎN TRATAMENTUL RĂNILOR: EVALUARE PRECLINICĂ *IN-VIVO*, ACTIVITATE ANTIMICROBIANĂ ȘI ASPECTE HISTOPATOLOGICE

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ABSTRACT | REZUMAT

Many records show that propolis has been used by ancient Egyptians, Persians, and Romans. New evidence suggests that propolis is as old as honey, Hippocrates, a Greek physician, considered the father of modern medicine, recognized the healing properties of propolis. An important factor in impaired wound healing is biofilm formation; propolis as an antimicrobial agent can reduce biofilm generation and result in an accelerated healing processes. In this study the aim was to develop a formula of a Propolis solution containing Propolis extract (30% Propolis tincture) which can assist in the wound healing and to evaluate the effects of this propolis formula on the healing process of wounds in rabbits by scrutinizing the *in-vivo* healing properties, antimicrobial activity and monitoring the histopathological aspects, and to suggest the most promising concentration for clinical use. Three propolis solution concentrations (1%, 2% and 3%) were evaluated and compared to a control solution (saline). The results of this research established that all propolis solutions had beneficial impact on healing processes with the 1% solution having the best healing properties *in vivo* pre-clinical evaluation confirmed by antimicrobial testing and the histopathological exam. The Propolis formula 3% concentration present a spectacular decrease in contamination of the wound and ensured the best results in limiting the microbiological load of the cutaneous surgically induced defects, but the Propolis formula 1% concentration induced the less regenerative side effects like acanthosis, fibroplasia, hyperkeratosis or rete ridges compared to the control group treated with saline.

Keywords: propolis, wounds, leporid, healing, evaluation

Multiple studii arată ca propolisul a fost un remediu ancestral folosit de către egipteni, persani și romani. Studii recente menționează că, încă din vremea lui Hipocrate, părintele grec al medicinei moderne, propolisul și mierea de albine erau recunoscute pentru proprietățile cicatrizante ale rănilor. O cauză importantă a tulburărilor de vindecare a rănilor este formarea biofilmului bacterian; propolisul ca și agent antimicrobian reduce formarea și generarea biofilmului având ca rezultat accelerarea procesului de cicatrizare. Scopul acestui studiu a fost de a elabora formula unei soluții pe bază de extract de Propolis (tinctură de propolis 30%), care să fie benefică procesului de vindecare al rănilor și monitorizarea efectelor acestei formule în procesul de vindecare a rănilor la iepure, prin evaluarea proprietăților de cicatrizare *in-vivo*, a activității antimicrobiene și evidențierea aspectelor de regenerare tisulară, care să permită recomandare formulei cu cele mai promițătoare efecte, benefice uzului clinic. În cadrul studiului au fost elaborate și testate trei formule pe bază de propolis (1%, 2% și 3%) care au fost comparate cu soluția salină fiziologică. Rezultatele acestui studiu au arătat că toate soluțiile de Propolis au efect benefic asupra cicatrizării rănilor, soluția 1% remarcându-se prin cele mai bune efecte în procesul de vindecare *in-vivo*, confirmate prin teste microbiologice și examen histopatologic. Soluția Propolis 3% asigură o reducere spectaculară a contaminării rănilor, asigurând cele mai bune rezultate în reducerea încărcăturii microbiene a rănilor induse operator, dar soluția Propolis 1% induce cele mai reduse efecte secundare regenerative (acantoză, fibroplazie, hipercheratoză), comparabile cu cele din rănilor tratate cu soluție salină fiziologică.

Cuvinte cheie: Propolis, răni, leporide, cicatrizare, evaluare

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Propolis and other honey bee products and subproducts have been used in cutaneous wound healing since the ancient period of the Mesopotamian, Egyptian, Roman and Persian civilizations, due to their therapeutic effects, including antimicrobial, anti-inflammatory, and

cell-stimulating properties (3, 12, 13, 14, 19, 21). A significant number of scientific research works that investigate the clinical efficacy, safety, and side effects of Propolis allowed for the development of novel products and clinical practices that are currently used by the clinicians and surgeons in the treatment of different types of traumatic skin injuries. Several therapeutic activities have been advocated, such as the antimicrobial (antibacterial, antiviral, antimycotic, antifungal), anesthetic, antioxidative, antiseptic, anti-inflammatory, antiulcer, spasmolytic, immunomodulatory, and healing properties (6, 8, 12, 15, 22). Wound repair and regeneration proceeds via a finely tuned pattern of integrated phases, such as hemostasis, inflammation, cell proliferation, and tissue remodeling, which all involve a number of cellular and molecular sequences (16).

The scientific evidence about the healing properties of propolis has increased, although the number of *in vivo* preclinical studies that investigate its healing properties in animal models and humans is limited (10, 13, 15).

The healing mechanism and properties of propolis remains a controversial issue, though this characteristic is likely due to the synergetic effects between the chemical constituents and its antibacterial and anti-inflammatory activities (12, 20).

The adverse reactions related to the use of propolis in wounds are poorly documented in the literature, and it is also critical to investigate the therapeutic levels and the cytotoxic concentrations of propolis products - in both *in vitro* and *in vivo* studies in order to guarantee its safety and to identify possible side effects (3, 23).

MATERIALS AND METHODS

A leporid model was used to accomplish the aim of the study. To investigate the Propolis solution effects on healing processes we used groups of 3 rabbits treated with Propolis formula and saline solution and 1 rabbit as a control. To study the propolis properties on the wound healing process, the investigators sought to create standardized conditions, in order to have results that would be comparable.

The study was performed on purpose-bred laboratory rabbits, in compliance with the European Union legislation concerning humane animal treatment and welfare of laboratory animals (Animal Protection Act §7-9; EU Convention on the protection of animals revised directive [86/609/EEC] included). Throughout the study, all legal and ethical requirements with regard to the humane treatment of animals were fulfilled.

A tincture of propolis 30% (called „mother” solution) was used to prepare the three solutions of 1%, 2% and 3% by diluting the „mother” solution with saline. Table 1 provides details on the mixture in the solutions which were obtained from the tincture of propolis (Fig. 1).

Table 1

**Propolis formula solutions
for *in-vivo* preclinical evaluation**

	1% propolis solution	2% propolis solution	3% propolis solution
Quantity of 30% propolis solution	0.33 mL	0.67 mL	1 mL
Quantity of saline added	9.67 mL	9.33 mL	9 mL
Final quantity	10 mL	10 mL	10 mL

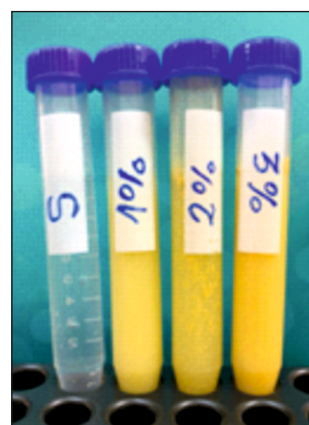


Fig. 1. Propolis 1%, 2%, 3%, and saline solution samples

The anesthetic protocol used during the surgery was as follows:

- *Premedication*: Butorphanol 0.2 mg/kg IM; Diazepam 1 mg/kg IM; Medetomidine 0.2 mg/kg IM, five minutes after;
- *Induction and maintenance*: Ketamine 20 mg/kg IM five minutes after;
- *Anesthesia reversion*: Atipamezole (5 times the dose of medetomidine).

On each of these rabbits, surgical defects or cutaneous wounds were induced, symmetrically to the median dorsal spine line on the thoracolumbar area. Two types of cutaneous defects, different in shape and sizes, were created in order to evaluate the properties of propolis in wounds healing:

- Defects performed by a biopsy trocar of 5 mm;
- Defects performed by surgical scissors inducing a larger defect.

The skin defects were made in two vertical rows, symmetrically: in cranial-caudal direction, on the left,

four surgical skin defects were created and on the right four cutaneous wounds by biopsy trocar (punch trocar) were made (Fig. 2).

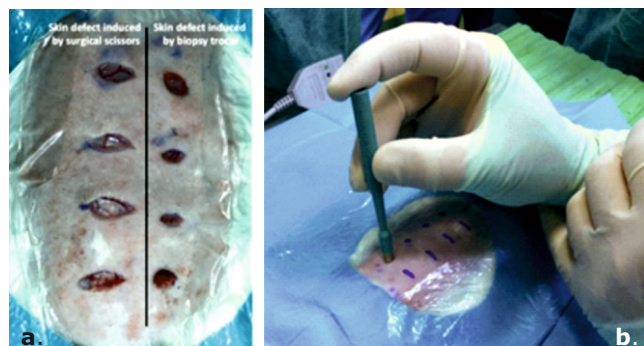


Fig. 2. Cutaneous defects:
a. cutaneous defects distribution
b. skin defects with punch trocar

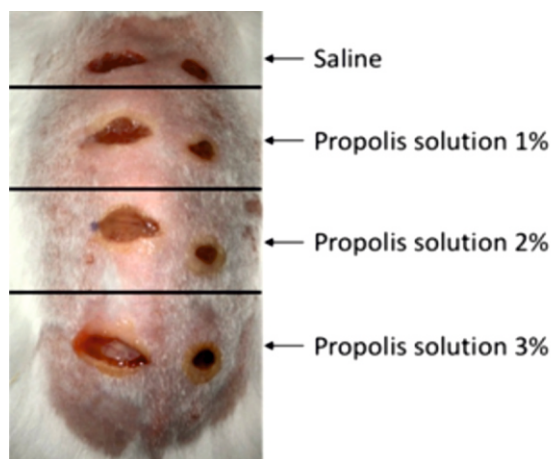


Fig. 3. Propolis solution application on the cutaneous defects

The cutaneous defects were treated daily with solution of a specific Propolis concentration (1%, 2% and 3%). The solutions were applied by means of a Q-tip in order to avoid contamination of the wounds by a solution of another concentration. The treatment of the wounds was conducted daily for each rabbit until full healing was observed (Fig. 3).

The appearance of the granulation tissue was carefully monitored and any abnormalities were recorded daily (2). Close-up monitoring the end of proliferation phase were evaluated.

The epithelialization presence and the progression of the wound contraction, which culminates with the closing of the wound, mean the end of the healing process.

To assess our hypotheses, we performed microbiological sampling of the surgically created wounds, intraoperatively (Day 0), two days later (Day 2) and four days later (Day 4), the swabs have been inoculated on blood agar and incubated over the night at 37°C.

The microbiological plates were monitored for colonies grown after 24 hours of incubation, to identify if bacterial contamination from the wound could be detected.

To fulfill the goals of this study, skin samples were harvested from the healed wounds to assess them at a microscopic level, by histopathologic evaluation.

RESULTS AND DISCUSSION

The three rabbits groups under scrutiny in this study were treated daily with propolis solutions of different concentrations (saline or 0%, 1%, 2% and 3%).

No other treatments were applied beside daily application of these propolis solutions on the skin wounds.

The wound aspects were checked on a regular basis by macroscopic evaluation by the same person, in order to achieve a subjective evaluation of the wound healing process.

To assess the wounds, the examiner focused on the time of the first appearance of the granulation tissue into the wound, and filling of the entire wound by granulation tissue up to the level of the surrounding skin (2). The examiner also concentrated his attention on the presence of abnormal secretion (exudate) into the wounds and the presence of secondary injuries from self-scratching, with delayed process of healing (Table 2).

The time to complete healing varied depending on the subject. The mean time for complete healing was 10 days after the surgical induction of the cutaneous wounds, that represented 9 days after the initiation of the treatment (2) (Fig. 4).

Table 2
Results of the wounds examination and granulation tissue appearance

	First appearance of granulation tissue		Filling of the entire wound by granulation tissue		Presence of abnormal secretion
	Trocar induced wounds	Surgical induced wounds	Trocar induced wounds	Surgical induced wounds	
Rabbit 1	Day 2	Day 3	Day 8	Day 9	Never observed
Rabbit 2	Day 2	Day 3	Day 9	Day 10	Never observed
Rabbit 3	Day 2	Day 3	Day 9	Day10	Never observed

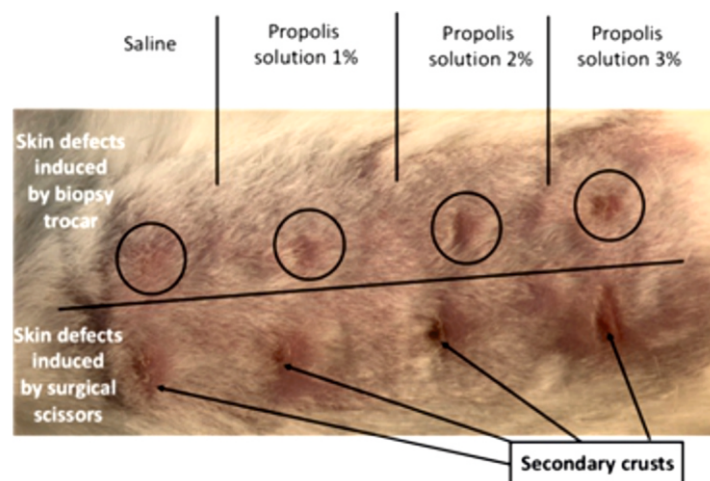


Fig. 4. Wounds aspects 10 days postop, treated with Propolis

Our microbiological results showed that propolis was clearly reducing the level of the microbial contamination at the wounds level. In this case, the solution that appeared to be reducing the most the microbial level was the 3% solution, wounds treated with 1% and 2% solutions were still presenting lower microbial load compared to the control group of wounds treated with saline. An important factor in impaired wound healing is biofilm formation. Biofilm consists of microbial communities of bacteria, fungi and others microorganisms. By affecting one or more phases of wound healing, the biofilms can delay the healing response. Propolis can be considered as a suitable biomaterial in

treating the wound biofilms. On the other hand, many studies have confirmed a synergistic potential between antibiotics and propolis and propolis may be a useful adjunctive agent in the acceleration of the antibiotic-resistant organisms (5, 9, 18). As expected, the results obtained from the sample swabbed during the surgery, were negative at 24 hours (day 0) for all the rabbits. 48 hours (day 2) after the surgery, the sampling and inoculated swab from the open wounds, revealed that the contamination of the wounds treated with saline is 6 to 9 times more significant than the contamination of the wounds treated with 1% or 2% propolis solution and approximately 18 times more pervasive than for the skin defects treated with 3% propolis solution.

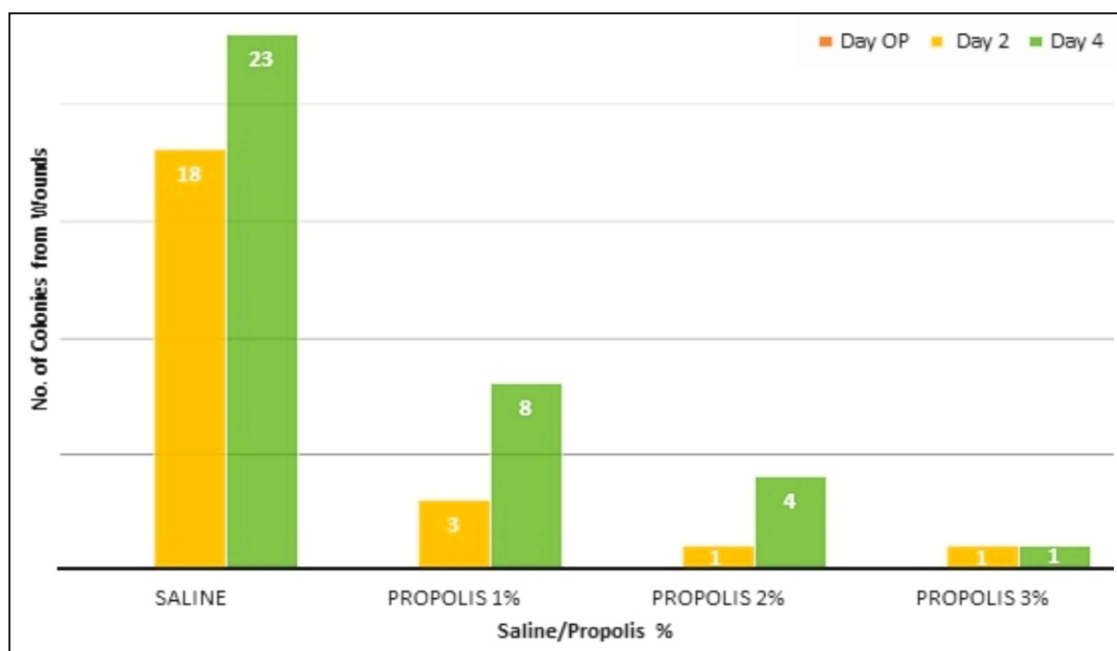
96 hours later (day 4), just like in the case of the results obtained 2 days following surgery, we observed a reduced contamination for the swabs sampled from wounds treated with propolis compared to the samples treated only with saline solution. For the wounds treated by 1% propolis solution we have a decrease in contamination ranging from 2 to 3 times.

The microbiological load of the wounds treated with 2% propolis solution is 4 to 5 times less important than the control group flora.

The samples taken from wounds treated with 3% propolis present a spectacular decrease in contamination amounting to a 23-fold decrease (Table 3).

Table 3

Propolis antimicrobial activity on the wound flora



The subjective macroscopic evaluation of the healing state of the wounds seemed to reveal that wounds treated with the propolis solution presented the fast-test healing. Following the skin regeneration, the fibroblastic regeneration observed in the dermis was much more discrete by comparison to the control group, presenting scattered inflammatory cells, basically plasma cells, but no neutrophils.

The epidermis in the healed region is much thinner than in the control group without hyperkeratosis. The dermis of the healed region presents numerous skin annexes (hair follicles and sebaceous glands).

All these features are characteristics that prove better properties of the Propolis 1% solution in the healing process (Fig. 5) and the induction of less regenerative side effects. The wounds treated with Propolis 1% healed the fastest. It has been shown that at higher concentrations (300 µg/ml), propolis directly activates mast cells and promotes release of the inflammatory mediators (7, 16)

In a model of study on Wistar rats, epithelisation concluded seven days after the injury. The increased healing activity has been attributed to increased collagen formation and angiogenesis (1, 4, 11).

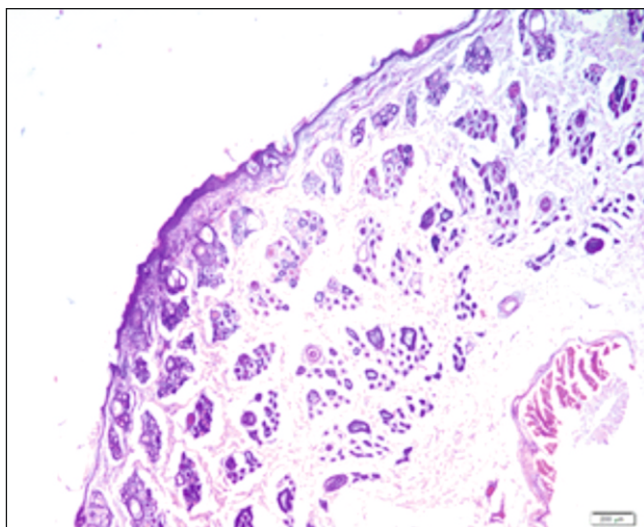


Fig. 5. Discrete dermal fibroplasia and acanthosis during healing treated with 1% propolis formula

If one compares the results obtained for wounds treated with 2% and 3% propolis solutions, it can be noticed that the results are similar, but with more acanthosis, fibroplasia and hyperkeratosis for the samples treated with the 3% propolis solution.

Animal studies showed the ability of propolis to promote the keratinocyte proliferation, the stimulation of glycosaminoglycan deposition in the wound, and the

modification of the chondroitin / dermatan sulphate structure (15, 17). Pessolato et al. (2011) reported the efficacy of a propolis ointment on the healing process of second-degree burn wounds by promoting wound debridement, stimulating the collagen synthesis, and reducing the wound inflammation (13).

CONCLUSIONS

In the *in vivo* pre-clinical study of surgically induced wounds, we concluded that complete wound healing was achieved 10 days after the surgical induction of the cutaneous wounds, which represented 9 days after initiation of the treatment. The histopathologic assessment of the cutaneous defects treated with propolis solution of different concentration (0%, 1%, 2% and 3%) revealed a reduced inflammatory aspect of the wounds regardless of the concentration used compared to the control (saline) solution. The Propolis formula 3% concentration presents the most spectacular decrease of wound contamination, and ensured the best results in limiting the microbiological load of the cutaneous surgically induced defects, but Propolis formula 1% concentration induced the less regenerative side effects like acanthosis, fibroplasia, hyperkeratosis or rete ridges compared to the control group treated with saline. Focusing on the healing quality aspect of the wounds treated with propolis, according to these results, the best concentration that should be used for therapeutic clinical activities is 1%. This concentration provides the best clinical relevance according to the healing features at a microscopic point of view and with the most effective antimicrobial Propolis concentration.

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