

Formulation and Wound Healing Evaluation in vivo of Ointment Based on Propolis, and Containing Curcumin

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ABSTRACT

Antimicrobial drugs are widely used in commercial ointments to treat different types of wounds. Unwise use of antimicrobials in ointment formulations only deepens the problem of antimicrobial resistance. Some natural products and plants (*Aloe vera*, honey, turmeric paste, garlic, and coconut oil) also possess antibacterial, anti-inflammatory and antioxidant properties. They are able to accelerate the healing of wounds. These wound-healing agents have the ability to suppress the development of pathogenic microflora on the wound surface, prevent the appearance of rotting of damaged tissues, and also have a weak analgesic effect. The aim of the study is to develop ointment formulations based on natural products and assess their physical properties, efficiency and safety for animals. Ointment formulations were prepared by fusion technique using animal fat, propolis, starch and curcumin. Physical evaluation of ointments pH, appearance, spreadability and stability was studied. Skin irritation and wound healing tests were applied on guinea pigs (*Cavia porcellus*). The first ointment contains 20% of Propolis and 5% of starch. The second ointment additionally hold 5% of curcumin. Ointments' pH was within the optimal limits (6.5±1.3 and 5.6±0.13). Both ointments showed good stability and perfect spreadability (121±3.5 and 112.4±2.6). The microscopic observation confirms higher spreadability of the ointment with curcumin. The ointments caused no irritation for the skin. Propolis ointment enhanced wound closure in male guinea pigs. Propolis+curcumin have great anti-inflammatory activity. The results showed that propolis and curcumin ointment is not irritable and can be used for wound treatment and healing.

Keywords: Curcumin; Ointment; Propolis; Wound healing

INTRODUCTION

Ointments are used widely in medical practice for therapeutic, prophylactic, diagnostic and cosmetic purposes. Antibiotics (bacitracin, neomycin, polymyxin, etc.) are common components in topical medicinal products for wound healing. Some commercial ointments incorporate natural products (propolis or curcumin) with anti-inflammatory and antimicrobial properties. Propolis is a natural product from various plants mixed with beeswax and salivary enzymes of bees (Fedoruk et al, 2015). Beeswax has antifungal properties and used as products covering for the preservation and prevention of fungal contamination (LiverTox, 2022). Propolis used in ointment formulations for the treatment of wounds, skin ulcers and rashes. It has antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antiproliferative and antioxidant properties (Przybyłek and Karpiński, 2019). Propolis is used both in traditional and complementary medicine to treat wounds due to its antiseptic and local anesthetic

characteristics. The ancient Egyptians, Greeks, and Romans used propolis for wound healing and disinfection (El-Sakhawy et al., 2024). Propolis is non-toxic and safe for human and animals. Propolis contains of different chemical compounds: resins, waxes, polyphenols, terpenoids, micro-, macroelements and vitamins. It is known to be well tolerated by human or animal organism. Rare allergic reactions can be caused by plant flavonoid aglycones (LiverTox, 2022). Chemical diversity of propolis is a key factor in combating antimicrobial resistance. Propolis directly acts against bacteria and also indirectly stimulating immune system (Przybyłek and Karpiński, 2019; Kapare et al., 2023). It is reported that propolis stimulates wound healing on various wound models *in vivo*. However, there is an information gap about appropriate dose, possible side effects and effectiveness of propolis on wound healing in human and animals (Oryan et al., 2018).

Some commercial wound healing ointments contain curcumin. Curcumin also has antibacterial, antifungal, wound healing, immunomodulating and other activities (Niranjan and Prakash, 2008; Yue et al., 2010). Researchers from various scientific fields are interested in the compound.

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Nearly 50 manuscripts devoted to curcumin are published each week (Nelson et al., 2017). Curcumin is a natural product (bright yellow powdered rhizome) originated from *Curcuma longa*. Curcumin is used in fresh wounds healing, applied to bruises and insect bites. Turmeric paste facilitates scabbing in chicken pox and small pox. Curcumin in different formulations are used for dermatitis and cutaneous infections treatment. Curcumin is stable in an acidic environment (Wang et al., 1997). It is insoluble in aqueous medium. Curcumin-polymer conjugates resolve the problem via covalent linking with water soluble polymers (Sood et al., 2023). Starch improves water-solubility of compounds forming a gel-like structure in water excess (Li et al., 2024). A hydrogel absorbs large amounts of water or biological fluid (Kapare et al., 2023). Starch is present in various plants. Maize and potatoes are main sources of starch for industrial applications (Sujka et al., 2018). Starch is a natural biopolymer that has many good qualities for pharmaceutical applications. It is used as binder, film-forming and drug delivery agent. Starch in ointments is used for skin disease treatments (Marto et al., 2018). Starch is also known for its wound-healing properties (Hassan et al., 2017) and used in skin tissue engineering research (Waghmare et al., 2018).

Wound healing is a complex process that has four phases: hemostasis and inflammation; proliferation with formation of granulation tissue; remodeling, with formation of new epithelium; scarring (Vollono et al., 2018). Curcumin enhances angiogenesis and reduces the epithelization period in wounds (Vollono et al., 2018). In the crisis caused by increasing number of microorganisms resistant to antibiotics, development of new effective ointment formulations with a multi-vector action is topical for skin healing. Both curcumin and propolis possess wound-healing properties and are used in various formulations separately. It's not common to introduce them in the same wound care product. We hypothesized that their combination in certain formulations could offer enhanced therapeutic effects.

The aim of the study was to study physical characteristics and biological effect of new ointments formulation with propolis, starch, and curcumin on guinea pigs' wounds.

MATERIALS AND METHODS

Materials

The materials used in this study were animal fat (Marcel, Romania), propolis (Megok, Ukraine), starch (Vimal, Ukraine), curcumin (Delux, India), ethanol (Sigma, Ukraine), chlorhexidine ointment (Unisan, Ukraine), digital

pH meter (ADWA, Hungary), analytical balance (EP64C "OHAUS", USA), microscope (Carl Zeiss, Germany), set of glassware (Sigma, Ukraine), scalpel (Troge medical, Germany).

Methods

Compounding

Propolis ointment 20%: a) 100 g of animal fat was melted in a clean enameled dish, bring to a boil, then remove from heat and cool to 50-60 °C; b) 20 g of crushed propolis cleaned of visible mechanical impurities and wax. Then propolis was added to cooled animal fat; the mixture was heated to 80°C, continuously stirring for 8-10 minutes (up to 2 hours). The dishes were covered; c) the obtained hot mixture was filtered through a gauze filter and cooled with constant stirring; d) the portion of the ointment was heated to 80°C, and to add 5 g of starch and 5 g of curcumin and cooled after filtration.

Physical characteristics

Color, odor and constituency were determined by the visual examination. The pH of the ointment was measured using digital pH meter. The pH meter was calibrated before measurement using buffered standard solutions with pH 4 and pH 7. Then they were washed with distilled deionized water and dried. The probe was immersed in the ointment, with care to ensure that the tip of the probe was completely covered by the ointment. After measuring the pH, the ointment was wiped off and the probe and rinsed sequentially with 70% (v/v) ethanol and distilled deionized water and dried.

The ointment spreadability was determined by placing the excess sample in between two glass slides which were compressed to uniform thickness by placing a 200 g weight on the top. The time required to separate the two slides was measured as spreadability (Mahaparale and Bauskar, 2019). Spreadability was calculated by the following formula:

$$S=M \times L/T$$

where S= Spreadability, M= Weight applied to the upper glass slide, L= Length of glass slide, T= Time taken to separate the slides.

Stability testing

The physical stability test of the ointment was carried out for four weeks at temperature conditions. The samples were submitted to heating at thermostat at 37 °C, to room temperature at 25 °C, and to cooling in refrigerator at 4 °C. Previously prepared formulations were stored at different temperature condition. Then physical

parameters (color, odor, constituency, pH and spreadability) were determined as described above.

Animals and procedures

Male guinea pigs (*Cavia porcellus*) that weigh 300–325 g were used in the study. Animals were kept in a pathogen free condition in vivarium on a standard diet and water ad libitum. Cutaneous longitudinal wounds of 5 mm were made on the pre-shaved, sterile (wiped with 70% alcohol) dorsal surface of the animal with the help of a scalpel. Three groups of animals were formed. Animals from the first group were treated with propolis ointment daily. Wounds on animals from the second group were treated with propolis+curcumin ointment daily for 5 days. Wounds on control animals (third group) were treated with commercial chlorhexidine ointment (Unisan, Ukraine) daily for 5 days.

Microscopic Evaluation

A small amount of the ointment was placed on the glass slide and spread evenly using the coverslip. The formulations were observed under a bright-field microscope using 40× objective, and photomicrographs were recorded (Manian et al. 2022).

Primary skin irritation assay

The test animals were acclimatized 5 days before the tests. Each guinea pig (*Cavia porcellus*) was kept in an individual cage in both temperature- and humidity-controlled environment. The ointment was applied to the pre-shaved clean skin in dorsal areas of guinea pigs (Kojima et al., 2021). The application sites on skin were observed for 24 hours.

Ethics Committee Approval

Ethics committee approval for this study was obtained from the Institute of Animal Biology NAAS, Lviv, Ukraine (Approval Reference Number: 154, 18.06.2024).

Statistical Analysis

Data analysis of ointment physical characteristics evaluation was determined by normality test using Microsoft Excel software. The values are presented as mean±SD of three determinations. Data is significantly influential if $p < 0.05$ and not significantly influential if $p > 0.05$.

RESULTS

Physical evaluation of ointments

According to Maru and Lahoti the organoleptic characteristics of an ointment include physical appearance, color, texture, phase

separation, homogeneity, and immediate skin feel of the ointment formulations. The organoleptic qualities of the ointments based on propolis and containing curcumin are shown in Table I.

Both ointments have smooth texture. They are opaque and homogeneous without grittiness or greasiness. The formulations are comfortable for the skin. They have different shades of yellow color and specific pleasant odors. pH of the formulations was found to be between 5.50 ± 0.2 and 6.5 ± 0.2 that is within the range comfortable to the skin (Lukić et al., 2021).

The spreadability test is a key characteristic of ointments and other topical formulations. Adequate spreadability ensures delivery of active ingredients, effectiveness, user satisfaction (Oktaviasari & Zulkarnain, 2017). The spreadability test showed that both ointments possessed great spreadability.

$$S1 = 200 * 1.5 / 2.71 = 110.7$$

$$S2 = 200 * 2.1 / 3.5 = 120$$

Propolis ointment spreadability is 120. The addition of curcumin lowered spreadability to 110.7.

Stability testing

The purpose of stability testing of ointments is to ensure that the product meets the intended physical and chemical quality standards, functionality and aesthetics. Physical and chemical stability studies of the ointments were carried out at 2 ± 2 °C, 25 ± 2 °C and 37 ± 2 °C for a period of 4 weeks (Table II). Both ointments had stable pH value, and spreadability. There were no phase separation, color and odor changes when stored under different 2 ± 2 °C, 25 ± 2 °C and 37 ± 2 °C temperature conditions.

Microstructure Observation

The microstructure analysis of a dispersed multi-phasic systems, including ointments, helps to understand how various components are combined and interact with each other (Chow et al., 2023). Figure 1 shows the microstructure of propolis ointments at 400 magnifications. Propolis ointment has very porous structure. The microstructure analysis confirms homogeneity of propolis ointment.

The propolis ointment with curcumin has more density structure than the one without curcumin (Figure 2). The microscopic observation confirms higher spreadability of the ointment with curcumin compared to the ointment that contains only propolis. The number of pores is significantly reduced both in number and size.

Table I. Physicochemical evaluation of ointment formulations

Ointment	Physical appearance	Colour	Texture	Phase separation	Homogeneity	Immediate skin feel
propolis	Opaque	yellow	Smooth	No	Homogeneous	No grittiness or greasiness
propolis+ curcumin	Opaque	bright yellow	Smooth	No	Homogeneous	No grittiness or greasiness

Table II. Stability study of ointment formulations after four weeks

Ointments	pH			Spreadability		
	4 °C	25 °C	37 °C	4 °C	25 °C	37 °C
propolis	6.5±0.97	6.5±1.3	6.4±0.96	118.2±2.7	121±3.5	124±3.2
propolis+ curcumin	5.5±0.78	5.6±0.13	5.6±0.43	109.1±1.9	112.4±2.6	112±3.6

*All values are mean±SD of three determinations. SD: Standard deviation

Ethics Committee Approval: Ethics committee approval for this study was obtained from the Institute of Animal Biology NAAS, Lviv, Ukraine (Approval Reference Number: 154, 18.06.2024).

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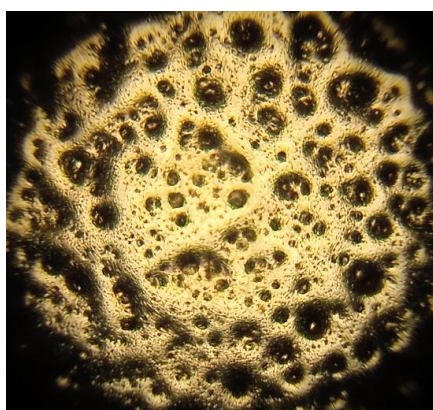


Figure 1. The structure of ointment that contains propolis (20%). Microscopy 400 magnification.

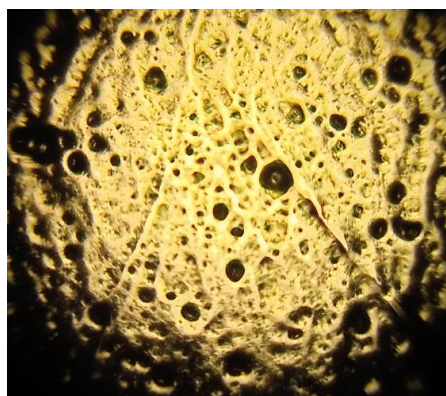


Figure 2. The structure of ointments with Propolis+curcumin. Microscopy 400 magnification.

Wound healing properties

Figure 3 shows the images of wound areas in guinea pigs and state of wounds at the beginning of the experiment, on the third and the sixth day. Ointments were applied daily. Enhanced wound closure was observed in the propolis and propolis+curcumin treated group (figure 3) on third day. Propolis+curcumin ointment facilitates scabbing. Propolis ointment also facilitates wound healing and possess anti-inflammatory activities. Propolis+curcumin also speed up wound healing and have anti-inflammatory activities. Both ointments prevent joining of microbial infection.

Wound sizes were significantly reduced after the application of propolis containing ointment on third and sixth day (figure 4). The treatment with propolis+curcumin ointment significantly reduced the size of wounds on sixth day.

Control group were treated with chlorhexidine commercial ointment. We observed irritation of wound on third day in control group. However, the irritation was absent on 6th day after ointment application and wounds were significantly reduced in size (figure 4).

Primary skin irritation assay

Some chemical or substance exposure may cause local inflammatory response to the skin. The 24-hour-exposed primary skin *in vivo* irritation test using rabbits, guinea pigs, mice and human volunteers is conventionally used for evaluating primary skin irritation (Kojima et al., 2021; Vinardell & Mitjans, 2017). Primary skin irritation

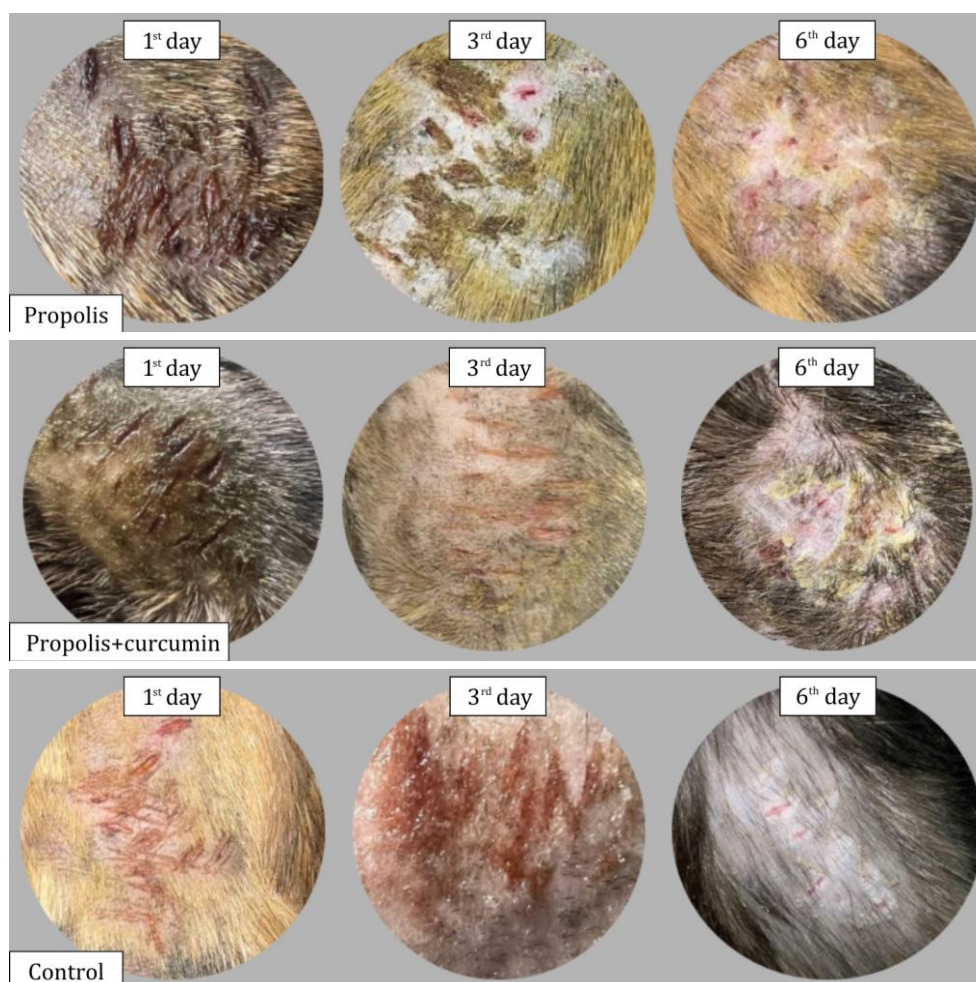


Figure 3. Representative image of guinea pig (*Cavia porcellus*) skin at the beginning of the experiment, on the third and on the sixth day.

testing was performed *in vivo* on guinea pigs. Both studied ointments did not irritate the skin surface. The signs of skin irritation such as redness, swelling, and blistering were completely absent in all tested animals.

DISCUSSION

The physical characteristics is crucial for the development of effective, safe and comfortable topical preparation. The results showed that both ointments are stable in low and high temperatures, have good spreadability. The pH of the ointments is safe for the skin.

Skin maintains the barrier function between the organism and the environment. It protects organism from infections and fluid losses (Díaz-García et al., 2021). Mechanical injuries are prevalent challenges in day-to-day lives of animals (Rennolds and Bely, 2023). In the condition of war in active phase, human and animals suffered from different mechanical and thermal wounds even

more (Prangenberg et al., 2021). The war and uncontrolled use of antimicrobial drugs promotes the increase in antibiotic resistant bacteria (Symochko et al., 2024). The concern around antibiotic resistance associated with antibiotic-containing topical preparations is growing (Blackburn, 2023). The effectiveness of antibiotics in ointments is low, especially for biofilm producing bacteria due to poor diffusion of antimicrobial drugs through the biofilm (Malik et al., 2017). Different bactericides are used for wound treatments. Unfortunately, they caused adverse reactions. It is reported that povidone-iodine had “protein error” effect in wounds characterized by high exudation (Stojko et al., 2021). Chlorhexidine inhibits tissue growth and delay wound healing (Stojko et al., 2021). Therefore, our aim is to develop effective alternative medicines for wound treatment using natural products with antimicrobial and anti-inflammatory characteristics. We chose propolis

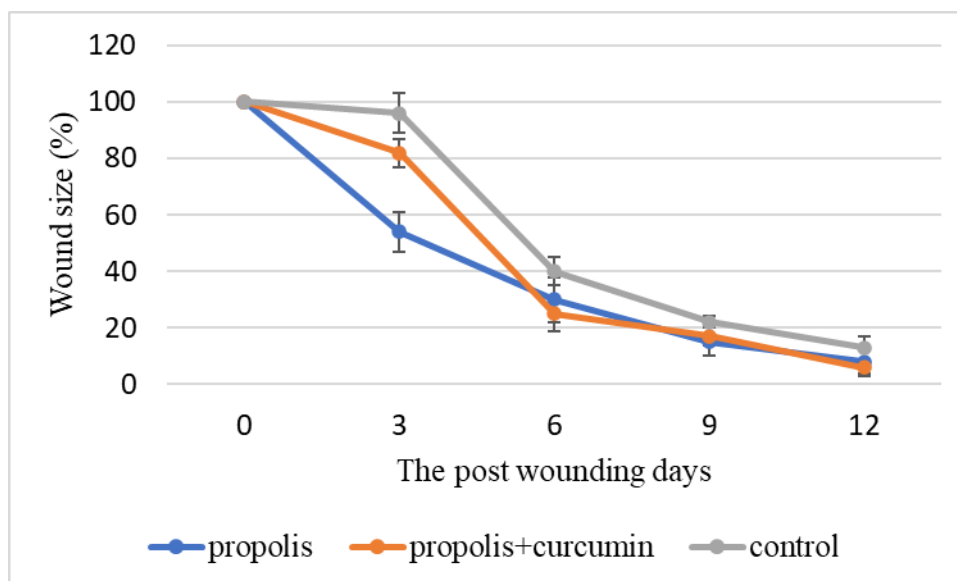


Figure 4. Wound healing curve of wound closure over the time period.

and curcumin that are known for their antibacterial and regenerative effects and starch as excipient.

Wound healing is serious therapeutic issue. Methods and medicines for speeding wound healing are constantly being explored (Stojko et al., 2021). Propolis is able to increase the expression of collagen on the early stage of wound healing. This trait helps to accelerate the wound closure (El-Sakhawy et al., 2024). We found that experimental ointments with propolis and propolis+curcumin significantly accelerate wound healing in guinea pigs (*Cavia porcellus*) by 42 ($p \leq 0.01$) and 14 % ($p \leq 0.05$) compared to commercial wound healing ointment with chlorhexidine. Chlorhexidine containing ointment delayed healing compared to experimental ointments with propolis and propolis+curcumin. Both experimental ointments with propolis did not cause acute irritation for human and animal skin. Propolis is able to damage the structures and function of bacteria cell wall. It also interacts with bacterial enzymes located in the cytoplasmic membrane, impacts nutrient transport, and the synthesis of cellular components (Stojko et al., 2021). Both ointments with propolis were efficient in preventing wounds from joined bacterial infections.

Propolis is also reported to decrease scarring (Stojko et al., 2021). We observed that ointments with propolis reduced scar formation and sped up the healing due to increased wound contraction and promoted tissue repair process.

Curcumin promotes wound healing accelerating angiogenesis and formation of

collagen fibers (Zhou et al., 2021). Curcumin also induces apoptosis of inflammatory cells and shortens the inflammatory phase (Barchitta et al., 2019). We observed reduced wound healing time after the treatment with propolis and containing curcumin ointment compared to the commercial chlorhexidine ointment.

We found that both experimental ointments are effective for wound healing and possess antiseptic properties. These ointments are considered a promising natural source for new pharmaceutical products for wound healing and wound reconstruction.

CONCLUSION

New ointments formulation with propolis, starch, and curcumin possess comfortable to the skin pH values, perfect spreadability and stability at different temperature conditions. The ointments are not irritabile for the skin. The data showed that the application of propolis and propolis+curcumin ointments accelerates wound healing in guinea pigs (*Cavia porcellus*). However, propolis+curcumin did not demonstrate well expressed enhanced therapeutic effect. Histopathology of skin should be performed in further research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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