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ORIGINAL RESEARCH

EXPLORING THE HEALING POTENTIAL OF PROPOLIS IN ORAL MUCOSAL INJURIES: A HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL EVALUATION

Aseel Kamil Hameed[1] , Alaa Abdulkhaliq Hussein[2] , Alaa Hussein Saadon[2]
[1]Department of Medical Microbiology, College of Dentistry, University of Basrah, Iraq.
[2]Anatomy and Histology Dept., College of veterinary medicine, university of Basrah, Iraq.
Corresponding author: Aseel Kamil Hameed Department of Medical Microbiology, College of Dentistry, University of Basrah, Iraq. Email Address: aseel.hameed@uobasrah.edu.iq Scopus ID: https://www.scopus.com/authid/detail.uri?authorId=57937593100
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ABSTRACT

Background: Propolis, natural resinous entity that bees build from plant exudates acts as anti-inflammatory, antioxidant; antimicrobial and regenerative. Based on its well-recognized therapeutic capabilities, there is limited knowledge related to the effect of propolis application towards promoting oral mucosal wound healing. The purpose of the present investigation was to study histochemical and immunohistochemical finding on the influence topical propolis gel upon oral wound healing in rabbits.

Methods: Forty-eight healthy adult male Iraqi rabbits (Oryctolagus cuniculus) were divided randomly into eight groups, four control groups (C3, C7, C14 and C21), survivored with propolis-free gel and four experimental groups (T3,T7, T14 and T21), survi vored with propolis-based gel. A standardized longitudinal cut (10 × 3 mm) was created in the hard palate of each rabbit. The healing process was monitored by measuring the extent of wound contraction. Masson trichrome staining was used to evaluate collagen fiber distribution and areas, fibroblast proliferation, and ECM organization. Moreover, immunohistochemical VEGF and TGF-β stainings were performed to detect angiogenic and regenerative capabilities.

Results: Wound closure (AB) was faster and the organization of extracellular matrix (ECM) fibers as well as collagen deposition were more optimally observed in propolis-treated than in control groups. Evidence of active angiogenesis and mucosal keratinization in the treated wounds was also noted at an earlier date. Moreover, VEGF expression was obviously upregulated ($P \leq 0.05$) in the irradiated T7, T14 and T21 groups as compared to that in their respective control ones, where a weak reactivity could be seen very localized.

Conclusion: Application of topical propolis gel had a remarkable perfective effect on the process of oral mucosal wound healing by a stimulatory activity of fibroblasts, promoting synthesis of collagen and facilitating angiogenesis. These results highlight propolis as a safe, bio-friendly biomaterial to enhance healing of oral soft tissues on dental applications.

***Keywords*:** Propolis, Oral wound healing, Immunohistochemistry, VEGF.

INTRODUCTION

Wound healing in the oral mucosa The process of wound repair within the oral cavity is an organized and bio-logically complex one that proceeds through multiple phases: inflam-mation, tissue proliferation and remodeling resulting in restoration of mucosal integrity and function. Mucosa of the oral cavity has remarkable regenerative potential that is owed to robust blood flow, basal/superficial cell turnover and continuous exposure to saliva containing antimicrobial peptides and growth-promoting substances[1]. The healing of such oral wounds, however, may be retarded due to infection or persistent inflammation as a result of either local- or system-based disorder that impedes normal cellular function. Accordingly, the need for biocompatible natural materials capable of accelerating healing while reducing complications in dentistry is on the rise[2] .

Propolis which is also known as “bee glue” are resinous material, collected by Apis mellifera bee from plant buds and exudates that mixes with beeswax and enzymatic secretions. More than 300 bioactive compounds, mainly flavonoids, phenolic acids, and terpenoids have been detected in propolis which contribute to its treatment versatility[3,4]

Its pharmacological properties include strong or wide-spectrum antimicrobial, antioxidant, anti-inflammatory and tissue-regenerative activities[5,6]. Since the oral environment is always exposed to microbial flora and mechanical irritation, these bioactive characteristics suggest that propolis might be a potential material for the oral cavity area wound healing[7]. Recently, propolis has been shown to modulate the inflammatory response and mediators Systemic diseases controlling fibroblast activity and angiogenesis (essential for successful tissue healing;[7,8]. Its flavonoids chrysin, galangin, and caffeic acid phenethyl ester (CAPE) are responsible for the reduction of oxidative stress and promote synthesis of extracellular matrix[4] . The upregulation of VEGF after the application of propolis also again highlights its important role in angiogenesis and fibroblast-repaired remodeling connective tissue [2,5] . Taken together, these actions accelerate granulation show the components as well tissue formation, epithelial regeneration and wound contraction, all of which are key parameters for a successful healing process.

Classical wound-healing methods in oral surgery were dominated by synthetic antimicrobial, antiseptic and antibiotic substances. Despite their efficacy, these approaches may cause side-effects and contribute to bacterial resistance but also hinder tissue regeneration [1,2] . In comparison, some natural products including propolis have potential biocompatibility, low side effects and excellent regeneration properties. In experimental reports, topical propolis showed increased keratinocyte migration, collagen fiber orientation and re-epithelialization resulting in better organization of healing[8].

While it is known that propolis has general biological properties, detailed histochemical, and immunohistochemical analyses of the drug systemic actions on the repair process in oral mucosa are lacking. Fibroblast proliferation, collagen organization and extracellular matrix structure can be visualized using histochemical techniques, whereas markers like VEGF are able to provide insights into the molecular aspects of angiogenesis and tissue remodeling[4,7] . The combination of these strategies gives a more complete view of the regenerative processes induced by propolis.

The current study was conducted to investigate the effect of topical propolis gel on the healing of oral ulceration in rabbits. The cellular and molecular effects of propolis gel were investigated by histochemical and immunohistochemical examinations in this study. It is expected that the results of this study lead to the development of natural biomaterials as supportive drugs for accelerated postoperative healing in dental and oral surgical settings.

MATERIALS AND METHODS

All experimental protocols were approved and performed in accordance with the guidelines of the Animal Ethics Committee at Faculty of Veterinary Medicine, University of Basrah, based on the international rules for laboratory animal care and use (National Research Council (US) 2011). Forty-eight healthy adult male autologous rabbits (Oryctolagus cuniculus) with body weight ranging from 1.8 to 2.2 kg were used in this study. The animals were kept in separate stainless-steel cages with controlled temperature (22 ± 2 °C), humidity (50–60%), and light/dark cycles of 12 hours, and fed on a standard pellet diet with ad libitum fresh water. A one-week acclimatization before the start of experiments was observed to reduce stress. The rabbits were divided randomly into eight groups of 6 each. Four groups were kept as controls (C3, C7, C14 and C21) treated only with basic gel without propolis addition.The other four treatment groups (T3, T7, T14 and 21) selections received topical applications with propolis-based gel daily.

The numbers were indicative of the number of days after incision at which the animals were killed (3, 7, 14 and 21). Randomisation followed the basic tenets of experimental design[9].Propolis gel preparation

Propolis gel was prepared with modifications as described by Kim et al. (2018). 2 g of Carbopol 934 was dispersed in 50 mL deionized water, stirring continuously until completely hydrated. Subsequently, 2 mL of propylene glycol and 25 g ethanolic propolis extract were added dropwise with continuous stirring. The volume of the latter was brought up to 100 mL with distilled water. The pH of the preparation was strictly fixed to 6.5–6.8 by adding drops of triethanolamine in order to secure both stability and biocompatibility with oral mucosa. A placebo gel was also used, prepared with the same excipients without propolis extract.

Anesthesia was induced with intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). A 10 × 3 mm standardized longi- tudinal incision along the hard palate midline was carried out under sterile conditions with a sterile scalpel. Immediately after cutting, the gel (with or without propolis) was topically administered. The treatments were administered once a day until the animals were sacrificed at their respective times[10]. The size of the wounds was recorded at each time point (3, 7, 14 and 21 days) to observe its contraction and assess healing process[11].

The animals were sacrificed at the indicated times, and tissue samples including the wound area and about 2 mm of edge non-tumour mucosa was excised. The specimens were then fixed for 24 hours in 10%

neutral buffered formalin, dehydrated through an ascending series of ethanol, cleared in xylene and embedded in paraffin. Sections of 4-5 µm thick were cut with a rotary microtome (Leica Biosystems, Germany).

Masson's Trichrome staining was performed following the modified procedure of Fischer et al. (2020) to evaluate the collagen fiber distribution and connective tissue organization. Blue indicated collagen fibers and red colored the muscle and cytoplasmic elements. For immunohistochemical analysis, 5-µm tissue sections were deparaffinized and rehydrated, and then subjected to antigen retrieval in citrate buffer (pH 6.0) at 95 °C for 20 min. The sections were subsequently incubated with 3% hydrogen peroxide to quench endogenous peroxidase. The sections were then kept in 4 °C for a night as the primary antibodies raised against VEGF (monoclonal, Abcam, UK; dilution 1:200). The slides were washed and incubated with HRP-linked secondary antibody, and the signal was developed using DAB as chromogen before counterstaining with hematoxylin. Stained cells under the light microscope (Olympus, Japan). Morphometric analysis was performed using ImageJ software (National Institutes of Health, USA) to measure epithelial thickness, percent collagen area, and VEGF-positive cells count per high-power field. Values are expressed as means ± standard deviation (SD). Statistical analysis of group- and time-related data was carried out using two-way ANOVA followed by Tukey's post hoc test in SPSS (ver. 23) (IBM Corp., USA). In case the normality assumptions were not fulfilled, we conducted equivalent non-parametric tests. $P < 0.05$ was considered statistically significant [12,13,14].

RESULTS

The histochemistry of the distribution and maturation was analyzed in terms of collagen fibers and on that basis the process of tissue healing was studied between T (treated) and C (control) groups at various stages during healing.

At C3, for control samples there was a partial cut of the mucosa and low level collagen under wound (Fig. 1).

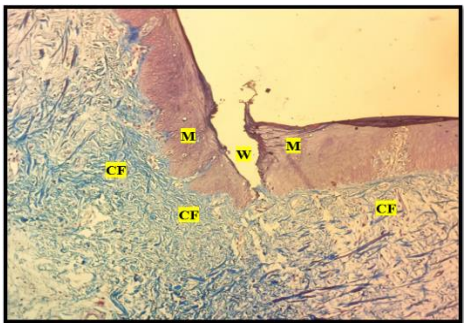

Figure 1. Histochemical micrograph of the incised hard palate of the C3 group shows open incised mucosa (M), there is low collagen fibers (CF) deposition beneath the wound (W) identified by the blue coloration. Masson's trichrome stain. 10X.

The Thyronorm T3 group on the other hand, showed weak density of collagen with initial entrance of new capillaries and mild inflammatory cell recruitment at wound margins (Figure 2).

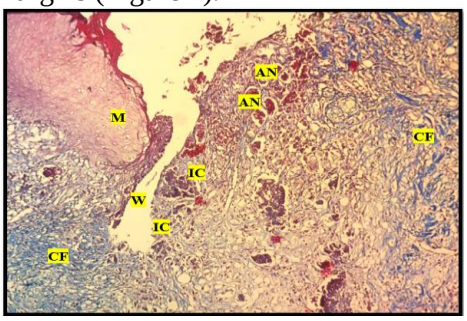

Figure 2. Histochemical graph of the incised hard palate of the T3 group shows open incised mucosa (M), there are low collagen fibers (CF) deposition beneath the wound (W) identified by the blue coloration; in addition, there is new blood vessels generation referred to active angiogenesis (AN) identified by the red coloration, as well as to infiltration of inflammatory cells (IC). Masson's trichrome stain. 10X.

By C7, control wounds were partially open with dense collagen deposition under the incision and visible neovascularisation (Figure 3).

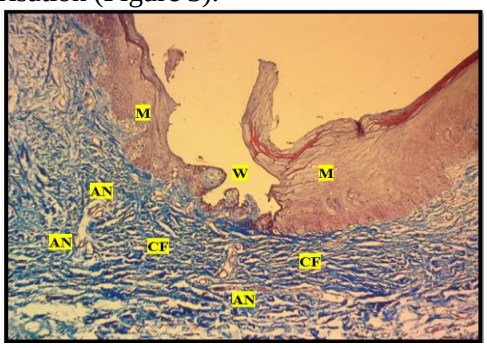

Figure 3. Histochemical graph of the incised hard palate of the C7 group shows open incised mucosa (M), there are dense collagen fibers (CF) deposition beneath the wound identified by the blue coloration, and new blood vessels generation referred to angiogenesis (AN) beneath the wound (W) identified by red coloration. Masson's trichrome stain. 10X.

On the other hand, in the T7 group, closer gap of wounds with denser and more regular bundles of collagen were observed that resulted active angiogenesis, alongside thickened keratinized epithelium suggesting an early proliferative activity (Figure 4).

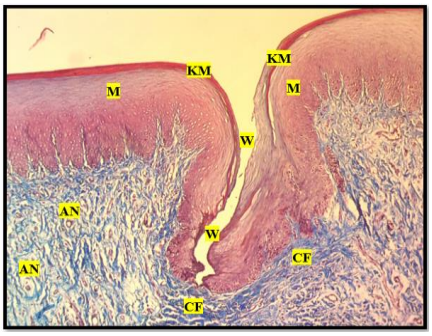

Figure 4. Histochemical graph of the incised hard palate of the T7 group shows narrow open incised mucosa (M), there are heavy and dense regular collagen fibers (CF) deposition beneath the wound (W) identified by the blue coloration. As well as blood vessel generation referred to as angiogenesis (AN) beneath the wound (W) identified by red coloration; also, there are thickened keratinized mucosal (KM) surfaces appeared in red color which refers to the early proliferation phase of wound healing. Masson's trichrome stain. 10X.

Control sections at 14 days after injury (C14) showed a retarded healing; the incision was still wide, the system of collagen was dense but disorganized, and an average angiogenic activity was observed (Table 5).

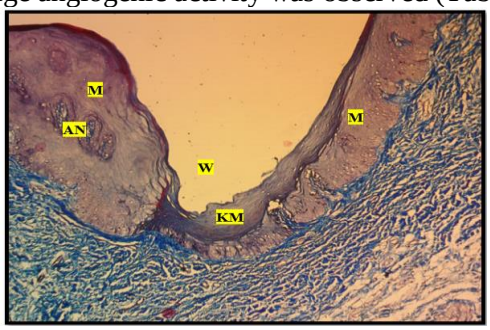

Figure 5. Histochemical graph of the incised hard palate of the C14 group shows wide open incised mucosa (M), there are dense regular collagen fibers (CF) deposition beneath the wound (W) identified by the blue coloration. As well as new blood vessel generation refers to angiogenesis (AN) in the mucosa; also, there are thickened keratinized mucosa (KM) appears in the wound site. Masson's trichrome stain. 10X.

On the other hand, the T14 group showed the full closure of epithelial layer associated with abundant and well-organized collagen fibers, a thickened keratinized mucosal layer; these results are indicative of advanced tissue remodulation and increased angiogenesis (Figure 6).

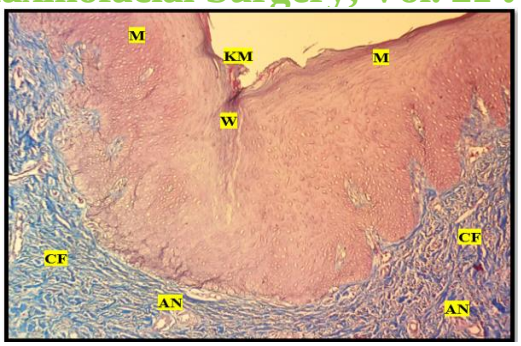

Figure 6. Histochemical graph of the incised hard palate of the T14 group shows closure of the incised mucosa (M), there are heavy dense regular collagen fibers (CF) deposition beneath the wound (W) identified by the blue coloration; also, there are thickened keratinized mucosa (KM) identified by red color which appears in the closed and completely healed wound site; as well as new blood vessel generation referred to angiogenesis (AN) beneath the healed wound. Masson's trichrome stain. 10X.

On C21, incomplete epithelialization was observed in the control group, and its wound was filled with densely oriented collagen, and inflammation infiltration around the ulcer area remained (Figure 7).

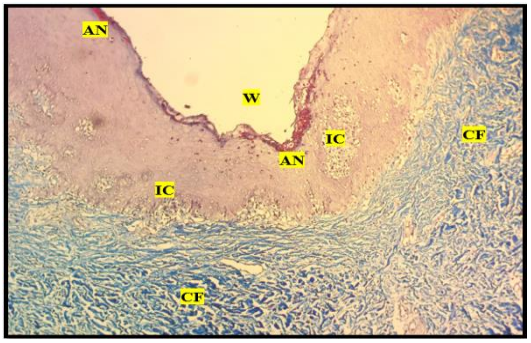

Figure 7. Histochemical graph of the incised hard palate of the C21 group shows partial closure of the incised mucosa (M), there are heavy dense regular collagen fibers (CF) deposition beneath the wound (W) identified by the blue coloration; also, there are new blood vessel generation referred to angiogenesis (AN) identified by red color; as well as to inflammatory cells (IC) infiltration in the mucosa. Masson's trichrome stain. 10X.

The T21 group showed completely closed wounds with thick, densely arranged collagen bundles, a continuous keratinized surface of the epithelium, evidence of late-stage angiogenesis indicating rapid remodeling and maturation of the tissue (Figure 8).

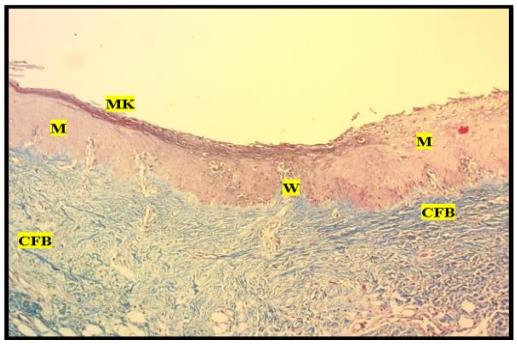

Figure 8. Histochemical graph of the incised hard palate of the T21 group shows complete closure of the incised mucosa (M), there are heavy regular collagen fibers bundles (CFB) deposition beneath the wound (W) identified by the blue coloration; also, there is a process of mucosal keratinization (MK) identified by red color referred to final and success remodeling phase of wound healing. Masson's trichrome stain. 10X.

- Vascular endothelial growth factor (VEGF) has been suggested to be important for the repair of tissues, primarily through promoting angiogenesis, collagen synthesis and epithelial regeneration. The stimulating effect of propolis on fracture healing were demonstrated indirectly by analyzing VEGF expression in the pre The symbol (*) indicates a significant ($p<0.05$) increase in the propolis treatment group compared to the control.

Quantitative analysis revealed a significant ($p \leq 0.05$) elevation in VEGF expression in the propolis-treated groups compared to their corresponding controls. Mean VEGF levels in T7, T14, and T21 were 71.05 ± 2.34, 79.93 ± 1.62, and 74.46 ± 1.85, respectively, while the control counterparts (C7, C14, and C21) showed markedly lower values of 30.41 ± 2.59, 34.97 ± 1.25, and 34.83 ± 2.64 (Figure 9).

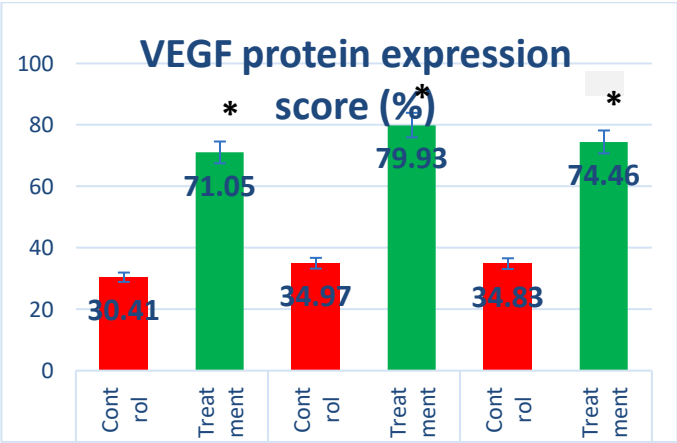

Figure 9. VEGF protein expression score (%)

Immunohistochemical localization demonstrated weak VEGF reactivity in the control groups, where positive staining occupied approximately 20% of the submucosal area in C7, increasing slightly to 30% in C14 and C21, with limited expression within the superficial epithelial layer (Figures 10, 11, and 12).

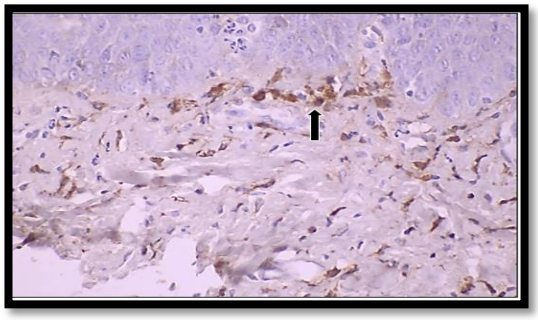

Figure 10. Photomicrograph of the hard palate of 7 days post-wound induction of control group; showed positive expression of VEGF protein (black arrow) was observed in the submucosal layer of the hard palate near the wound area, occupying approximately 20% of the dermis area. However, VEGF expression was not detected in the superficial mucosal layer. Hematoxylin and DAB. 400x.

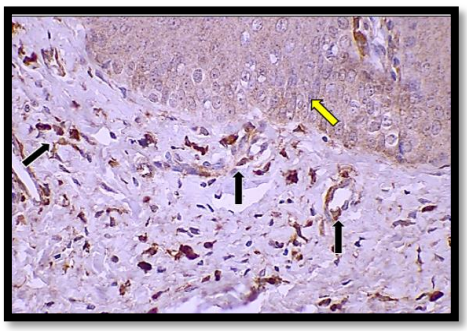

Figure 11. Photomicrograph of the hard palate of 14-days post-wound induction of control group; showed positive expression of VEGF protein (black arrow) was observed in the submucosal layer of the hard palate near the wound area, occupying approximately 30% of the area. However, VEGF expression was weak in the submucosal layer (yellow arrow). Hematoxylin and DAB. 400x.

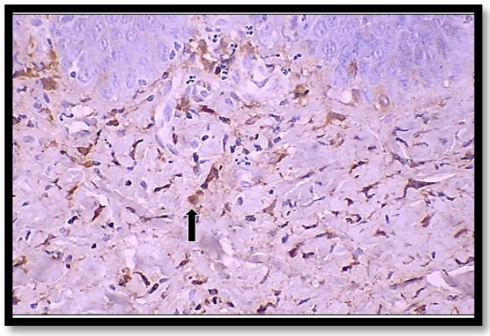

Figure 12. Photomicrograph of the hard palate of 21-days post-wound induction of control group; Positive expression of VEGF protein (black arrow) was observed in the submucosal layer of the hard palate near the wound area, occupying

approximately 30% of the dermis area. However, VEGF expression was weak in the superficial mucosal layer (yellow arrow). Hematoxylin and DAB. 400x.

In contrast, propolis-treated specimens exhibited intense VEGF immunopositivity extending throughout the submucosa. VEGF-positive areas occupied about 70% of the submucosal layer in T7, and approximately 80% and 70% in T14 and T21, respectively, indicating active angiogenic activity and accelerated wound repair (Figures 13, 14, and 15).

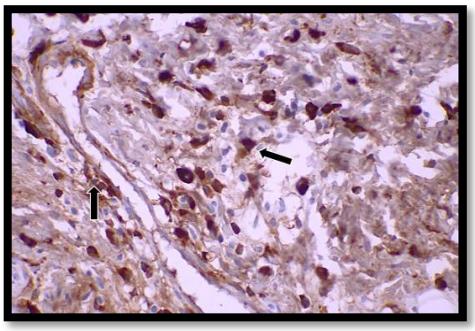

Figure 13. Photomicrograph of the hard palate of 7 days post-wound induction of propolis treatment group; Strong expression of VEGF protein (black arrow) was observed in the submucosal layer of the hard palate near the wound area, occupying more than 70% of the area. However, VEGF expression was not observed in the superficial mucosal layer. Hematoxylin and DAB. 400X.

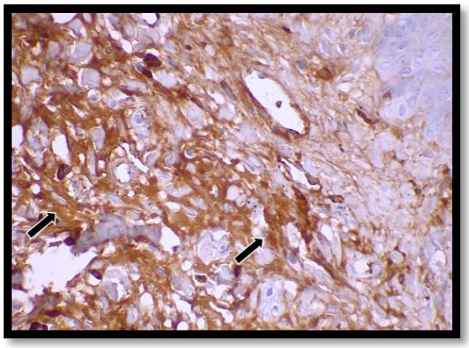

Figure 14. Photomicrograph of the hard palate of 14 days post-wound induction of treatment group; Strong expression of VEGF protein (black arrow) was observed in the submucosal layer of the hard palate near the wound area, occupying more than 80% of the area. However, VEG expression was weak in the superficial mucosal layer (yellow arrow). Hematoxylin and DAB. 400x.

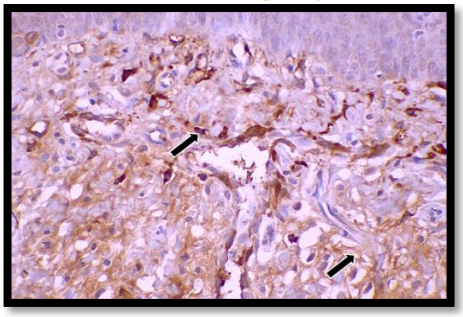

Figure 15. Photomicrograph of the hard palate of 21 days post-wound induction of treatment group; Strong expression of VEGF protein (black arrow) was observed in the submucosal layer of the hard palate near the wound area, occupying more than 70% of the tissue area. However, VEGF expression was weak in the mucosal layer (yellow arrow). Hematoxylin and DAB. 400x

DISCUSSION

The histochemical and immunohistochemical results of the current experimental model have demonstrated that the topical application of propolis could accelerate wound repair in oral mucosa by comparison with untreated controls. The healing process was assessed according to maturation of collagen fiber, regeneration of epithelium, and expression level of VEGF during different intervals for wound healing (3rd, 7th, 14th, and 21st day).

Formation and maturation of collagen are determining factors for the quality of wound healing process, giving tensile strength and sagacity structure for growing tissue [15]. During the study, histochemical staining showed a time-dependent deposition of collagen in both treated and nontreated wounds; however, it appeared denser and better organized in the propolis treated wound. At 7 days post-injury, treated sections demonstrated closely packed and well-aligned collagen bundles compared to the control specimens with loosely arranged collagen fibers and continued open wound.

This structural alignment of the collagen fibers in the propolis-treated tissues reflects increased fibroblastic activity and more effective extracellular matrix reorganization. These similar results have been found by Silva et al. (2020) who reported on fibroblast proliferation and hydroxyproline content, the factor for collagen synthesis enhancement, augmented by propolis[16]. Moreover, the compact and well-aligned arrangement of collagen fibers on days 14 and 21 in treated group indicates remodeling and maturation, reflecting that early healing was advanced than control.

In comparison, control groups showed delayed healing with irregular collagen formation and sustained inflammation. It is possible that such matrix remodeling in the matrix may contribute to delay

matrix turnover and collagen degradation due to absence of bioactive factors that encourage cellular proliferation and angiogenesis. Flavonoids, phenolic acids and terpenes are found to be present in high concentrations in propolis; these offer antioxidant and anti-inflammatory activities that reduce damage caused by tissue oxidative stress as well as increase collagen incorporation[17,18].

Angiogenesis is critical to successful wound healing as it facilitates the supply of sufficient oxygen and nutrients to reparative tissues. Vascular Endothelial Growth Factor (VEGF) is one of the main effectors which control neovascularization during tissue healing process[19]. The quantitative analysis of this study showed that there was a statistical difference ($p \leq 0.05$) in the VEGF expression by propolis groups compared to their respective controls, at all healing times.

Immunohistochemical results also supported these quantitative values. Strong VEGF immunopositivity, localized in the submucosal and epithelial layers was observed in the treated animals, mainly at 7 and 14 days. These findings are in line with those of Aboelsaad et al. (2022) and Aziz et al. (2025) found that the stimulation effect on VEGF expression of propolis application was due to the promoting role of its polyphenolic components on endothelial cell proliferation and migration[20,21]. The very strong angiogenic response seen in propolis-treated wounds also confirms the rapid enhancement of collagen maturation that was histochemically evidenced. The crosstalk between angiogenesis and ECM remodeling has been previously reported as enhanced vascularization in turn increases the fibroblasts and collagen synthesis[22,23,24]. On day 21, the presence of an closed epithelial layer and organized collagen alignment within the treated group provided evidence that propolis application can promote tissue maturation and healing in later stages.

The keratinized epithelial layer in treated wounds was obviously thicker and more continuous than that of the control group, which suggested a faster epithelial proliferation and differentiation. Such effects might be due to the presence of caffeic acid phenethyl ester (CAPE) in propolis that reportedly promotes keratinocytes migration and inhibits the release of proinflammatory cytokines[25,26]. The initial epithelial hyperplasia observed up to day 7 in the treated group, demonstrates the anti-inflammatory and anti-oxidant effects of propolis that promotes a suitable microenvironment for rapid mucosal healing.

The accelerated wound healing in the propolis treated groups may be due to its wide range of bioactive compounds. Propolis acts synergistically by modulating inflammatory mediators, inducing the proliferation of fibroblast and endothelial cells, and activating angiogenetic pathways by upregulating VEGF and transforming growth factor-beta (TGF-β)[16,27,28]. Also, its antioxidant capacity can scavenge free radicals causing inhibition of cell proliferation and collagen cross-linking. Overall, results of this study contribute to the previous findings on the wound-healing-promoting properties of propolis. Rapid epithelialization deriding its close apposition to the epithelium, dense aggregation of collagen and increased expression of VEGF highlight strong regenerative potential propolis in oral mucosa repair.

5.CONCLUSION

In conclusion, topical propolis could govern nonspecific migratory distance by increasing collagen maturity and vascularization through elevated expression of VEGF, as well as contributing to complete epithelial tissue regeneration with significant improvement in the rate of oral wound healing. These findings point out the propolis as a potential agent of interest for clinical application in oral surgery and tissue regeneration dentistry. Prospectives Studies using molecular markers and ultrastructural analyses may help to clarify its mechanisms, thereby potentially facilitating the targeted therapeutic application of it.

DECLARATIONS

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Conflict of Interest

The authors declare no conflict of interest.

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