COLLAGEN-BASED SYSTEMS WITH PROPOLIS TINCTURE FOR DERMAL TISSUE REGENERATION

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Burns are a widespread issue affecting many individuals, and their complications can lead to significant consequences. To mitigate these complications, it is advisable to substitute synthetic active ingredients with natural ones that offer various properties conducive to effective wound healing and prevent the formation of bacterial biofilms. This study focuses on creating systems from natural materials, specifically collagen derived from bovine skin and propolis, a product from bees, with antibacterial properties. The resulting systems were examined through several characterization techniques to assess their morphology, active ingredient content, release profile, and enzymatic degradation. The findings indicate that this natural component-based system shows great potential as a substitute for synthetic materials, offering the potential for effective dermal tissue regeneration and antimicrobial activity.

Keywords: burns, wound healing, dermal tissue regeneration, biomaterials, collagen, propolis.

1. Introduction

The skin serves as the main barrier between the body and the environment, playing a vital role in thermoregulation and protecting against pathogens. Skin injuries can pose serious health risks, as damage may compromise the epidermal tissue, vascular network, and, depending on the wound's depth, the complex dermal structure as well [1].

Burns are generally characterized as skin injuries caused by thermal shocks, electrical sources, chemicals, or radiation exposure. It is crucial to prevent and manage infections in these cases, while also facilitating effective healing [2].

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Scientific research aims to improve wound healing and the effectiveness of treatments by enhancing the antimicrobial activity of various therapeutic systems. The studies focus on facilitating wound disinfection and accelerating healing through tissue regeneration mechanisms [3, 4].

Conventional treatments for wounds often use antimicrobial agents like antibiotics, povidone-iodine, and silver sulfadiazine. Emerging strategies are exploring the use of antimicrobial molecules and therapeutic microorganisms, such as probiotics and bacteriophages [4, 5].

Natural products have long been used in traditional therapies, and their effects have been evaluated in various studies. Their cost-effectiveness, along with properties such as antimicrobial, anti-inflammatory, and antioxidant effects, has attracted researchers' interest as potential alternatives to modern products and therapies [6]. Natural systems are usually preferred as skin substitutes in wound healing due to their biocompatibility, non-irritation, non-toxicity, and ease of application [7]. Collagen is commonly used in composite systems to enhance scar repair from dermal injuries. Research has also focused on incorporating bioactive compounds into these collagen-based systems to allow controlled drug release, thereby promoting quicker granulation tissue formation and epithelialization [8, 9].

Collagen, the main protein in connective tissue, constitutes over 30% of the body's total protein mass and is essential for cell viability, scar formation, disease progression, and recovery [10]. It is also vital for skin health and, when used in wound treatments, stimulates fibroblasts and immune cells. This action helps protect the extracellular matrix and promotes wound healing [11]. When an injury happens, collagen activates and aggregates platelets, leading to fibrin clot formation at the site. It also recruits fibroblasts, epithelial, and endothelial cells during inflammation, aiding in tissue remodeling and the development of tensile strength. Collagen is present in the scar tissue formed during wound healing. Given its crucial role in this process, there is growing interest in using collagen for wound treatment [12]. Propolis has been utilized in traditional medicine for thousands of years. It is a natural mixture of resin, pollen, waxes, and enzymes gathered by bees from plant sources like poplar and willow. Bees use propolis to seal cracks, regulate hive size, maintain internal balance, and protect against intruders, decay, and environmental fluctuations. The composition of propolis is highly complex, containing over 300 identified chemical components, primarily rich in flavonoids, terpenes, phenolic acids, amino acids, hydrocarbons, minerals, trace elements, vitamins, and enzymes [13]. Propolis has therapeutic properties that aid in tissue repair and injury regeneration, attributed to its immunomodulatory, anti-inflammatory, and antimicrobial effects. It reduces free radicals in inflammatory injuries and promotes collagen production. Additionally, propolis accelerates various enzymatic reactions, enhances cellular metabolism and blood circulation, and supports

collagen fiber formation, thanks to its content of bioflavonoids, arginine, vitamin C, provitamin A, B vitamins, and minerals [14].

The purpose of this study is to create collagen-based biomaterial systems incorporating propolis tincture, primarily for dermal tissue regeneration, particularly in the treatment of burn wounds, and to characterize the resulting biomaterials through a series of comprehensive analyses. The addition of propolis in tincture form to the collagen matrix leverages the exceptional properties of this bee product, including its antimicrobial, antiparasitic, anti-inflammatory, healing, anesthetic, and antioxidant qualities, as well as its ability to boost antibody production. Propolis plays a vital role in scar repair.

2. Materials and methods

To develop collagen-based biomaterial systems incorporating propolis tincture, the following raw materials were utilized:

• Collagen Gel: was derived from raw calf skin using a patented method at the Collagen Department of INCDTP – Leather and Footwear Research Institute.

• NaOH (Sodium Hydroxide): Used to adjust the pH of the collagen gel.

• GA (Glutaraldehyde): Used as a cross-linking agent.

• Propolis Tincture (T): Prepared by dissolving 100 g of propolis from a personal apiary in 1000 mL of 90% ethanol and allowing it to macerate for 48 hours.

The procedure for preparing samples for the systems based on collagen and propolis tincture involved: a) Preparing a 1% collagen gel; b) Adjusting the pH of the collagen solution to physiological levels; c) Mixing the collagen gel with propolis tincture in various concentrations; d) Cross-linking the collagen-based samples; e) Producing spongy forms (sponges) through freeze-drying the final samples.

To prepare the hydrogel, we started with a 1% collagen gel to achieve a final volume of 170 mL. Starting with an initial pH of 2.20, we gradually added a total of 4365 µL of NaOH using a micropipette to achieve a final pH of 7.4. Subsequently, we added 8.91 mL of water.

Once the collagen gel was prepared, it was transferred into four Berzelius beakers: one served as the control sample with 40 mL of collagen, while the other three samples contained 35.6 mL collagen with 1% propolis tincture, 34.8 mL collagen with 3% propolis tincture, and 34 mL collagen with 5% propolis tincture. For cross-linking, 4 mL of GA(0.25%) was added to each sample. The collagen gels were prepared with varying concentrations of propolis tincture as detailed in Table 1. These codes and compositions will facilitate the identification and comparison of the different gel formulations in subsequent analyses.

To dry the obtained samples, a Delta LSC 2-24 lyophilizer (Martin Christ, Germany) was used.

Table 1

Composition and Coding of Collagen Gels

Sample	Collagen (1%), mL	Propolis tincture, mL	GA (0.25%), mL
Control sample	40	-	4
T 1%	39.6	0.4	4
T 3%	38.8	1.2	4
T 5%	38	2	4

The collagen and propolis tincture mixtures from the Berzelius beakers were poured into glass Petri dishes with a diameter of 5.2 cm and a height of 1.0 cm. These dishes were placed on the shelves of the lyophilizer, which had been pre-cooled to -40°C for 1.5 hours. Lyophilization was carried out for 48 hours in 11 stages as detailed in Table 2.

Table 2

Freeze-drying program of collagen hydrogels

Phase No	Freeze Drying Process Step	Time, hours	Shelf Temperature, °C	Pressure, mbar
1	Freeze *	1 ½	-40	-
2	Freeze dryer loading	¼	-40	-
3	Freeze *	3 ½	-40	-
4	Main freeze-drying	¼	-40	0,1
5	Main freeze-drying	4 ¾	-40	0,1
6	Main freeze-drying	10	+10	0,1
7	Main freeze-drying	8	+20	0,1
8	Main freeze-drying	12	+35	0,1
9	Final Freeze Drying	¼	+30	0,1
10	Final Freeze Drying	¾	+30	0,01
11	Final Freeze Drying	7	+35	0,01

* Manual freezing: phase 1 with empty shelves, phase 3 – with shelves full of samples [15]

The lyophilization (freeze-drying) process follows a structured program with specific time, temperature, and pressure settings for each phase:

Freezing Stages (Phases 1, 3, and 2 for loading):

- Maintains -40°C to solidify water content before drying (5 hours).

Primary Drying (Phases 4 to 8):

- Gradual temperature increase from -40°C to +35°C under 0.1 mbar vacuum to allow sublimation of ice (35 hours) .

Secondary Drying (Phases 9 to 11):

- Further moisture removal at +30°C to +35°C, with vacuum reduction to 0.01 mbar for product stabilization (8 hours).

Fig. 1 illustrates the appearance of the samples before and after lyophilization.

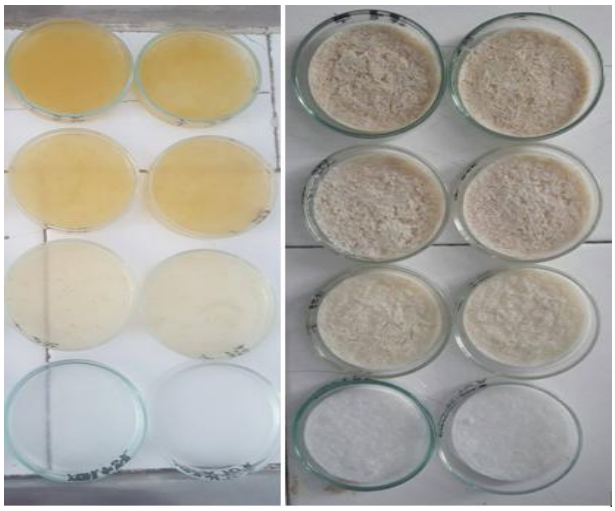

Fig. 1. Samples before lyophilization (left) and after lyophilization (right).

Lyophilization consists of a sequence of three distinct processes: 1. cooling below the freezing point; 2. sublimation of ice below the freezing point, typically performed under low pressure; 3. removal of residual, unconsolidated water from the solidified solution. The gels were lyophilized and the corresponding matrices were obtained, namely the sponges.

3. Characterization of the obtained systems

FT-IR: FT-IR spectra were recorded using a Fourier-transform infrared spectrometer (Bruker Vertex 70) coupled with a RAM II FT-Raman module, within the range of 600-4000 cm^{-1}, at a resolution of 5 cm^{-1}, and with 32 scans.

Differential Scanning Calorimetry (DSC): Non-isothermal DSC curves were obtained using a NETZSCH DSC 204 F1 Differential Scanning Calorimeter.

Approximately 10 mg of sample was heated at a rate of 10 °C/min over a temperature range of -20 to 300 °C, consisting of two passes.

Micro CT: MicroCT analysis of the microparticles was performed using a Skyscan 1272 X-ray microtomography system (Bruker MicroCT). The samples were secured to the sample holder with modeling clay and scanned at a magnification of 26× (where one pixel corresponds to a cubic voxel resolution of 4.95 μm) at 40 kV, 86 μA, with a rotation angle of 0.25°, no filtering, and an average of 4 frames. Approximately 1000 2D slices were obtained for each sample type using NRecon software (version 1.6.9.18, Bruker). 3D reconstructions were prepared using CTVol software (Bruker).

Enzymatic Degradation: To test the enzymatic mass loss of the collagen and propolis tincture matrices, samples were immersed in water for 48 hours, weighed, and then immersed in collagenase solution. The enzymatic mass loss of the samples was analyzed based on the exposure time to the Type I collagenase solution (0.1mg/ml). The 4 samples were analyzed in triplicate for better accuracy of the results. At well-established time intervals, namely 1h, 2h, 3h, 4h, 24h and 48h and in thermostatic bath (37ºC), the samples were removed from the degradation solution and weighed.

To determine the percentage of degradation of the samples, the following equation (1) was used:

$$Enzymatic\ Mass\ Loss\ [\%] = (Wi - Wt) / Wt \times 100 \quad (1)$$

Where *Wi* is the initial weight and *Wt* is the weight recorded at time *t*.

UV-VIS Release: The release profile of the active substance was determined using an automated dissolution bath, USP Apparatus 1 (708-DS Agilent), which includes an automated multi-channel peristaltic pump (810 Agilent), and a UV/VIS spectrophotometer (Cary 60) with a quartz cuvette featuring a 1 mm optical path, along with UV-Dissolution software. Samples were placed in a dialysis bag containing 8 mL of buffer solution and immersed in 200 mL of dissolution medium at 37 °C. The rotation speed was set at 75 rpm. The samples were maintained for 24 hours in a phosphate buffer solution at pH 7.2. At specific time intervals, the amount of propolis released was measured using a calibration curve at 292 nm. The calibration curve was generated using an etalon propolis solution prepared through serial dilutions, and the resulting slope was utilized to determine the concentration of released propolis based on the Lambert-Beer equation (2):

$$A = \varepsilon \times c \times l \quad (2)$$

Where A is absorbance of the solution (dimensionless, measured by a spectrophotometer), ε (epsilon) is molar extinction coefficient ($L \cdot mol^{-1} \cdot cm^{-1}$), a constant specific to each substance at a given wavelength, c is concentration of the

solution (mol/L) and *l* is path length of the cuvette (cm), representing the thickness of the solution through which light passes.

The calibration curve is used to determine the amount of propolis released from the analyzed samples and can be seen in Fig. 2.

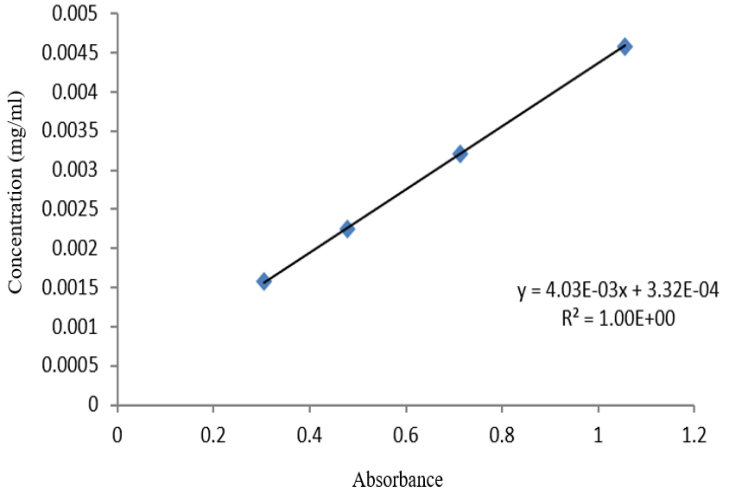

Fig.2. Calibration curve

The phosphate buffer solution at pH 7.2 was prepared by dissolving 9.075 g of potassium dihydrogen phosphate in water to produce 1000 mL of solution A and dissolving 11.87 g of disodium hydrogen phosphate in enough water to produce 1000 mL of solution B. Then, 300 mL of solution A was mixed with 700 mL of solution B.

4. Results and discussion

4.1. FT-IR Analysis

FT-IR spectroscopy was conducted to confirm the presence of propolis in the samples obtained. In the FT-IR spectrum (Fig. 3) of the control sample, specific collagen peaks can be identified. At 3318 cm^{-1}, the characteristic stretching vibration of the N-H bond from primary amines is observed. The peak at 2917 cm^{-1} is attributed to the C-H stretching vibration from amino acids. The C=O stretching vibration corresponding to Amide I from collagen appears at 1655 cm^{-1}, while the C-N stretching vibration of collagen Amide II is found at 1551 cm^{-1}. Wavenumbers between 1400 and 1200 cm^{-1} correspond to Amide III from collagen, and the peaks at 1081 cm^{-1} and 1025 cm^{-1} are associated with C-O-C stretching and C-O stretching vibrations, respectively.

In the FT-IR spectrum of the propolis tincture, peaks at 2927 cm^{-1} and 2848 cm^{-1} are observed, which correspond to the asymmetric and symmetric stretching

vibrations of the C-H bond found in hydrocarbons. The stretching vibrations of the C=O and C=C groups, as well as the bending vibration of the N-H group, were identified at 1635 cm^{-1} and 1511 cm^{-1}, indicating the presence of these groups in significant amounts within aromatic compounds, such as flavonoids. The bands at 1162 cm^{-1} and 1032 cm^{-1} belong to the aromatic rings of the flavonoids.

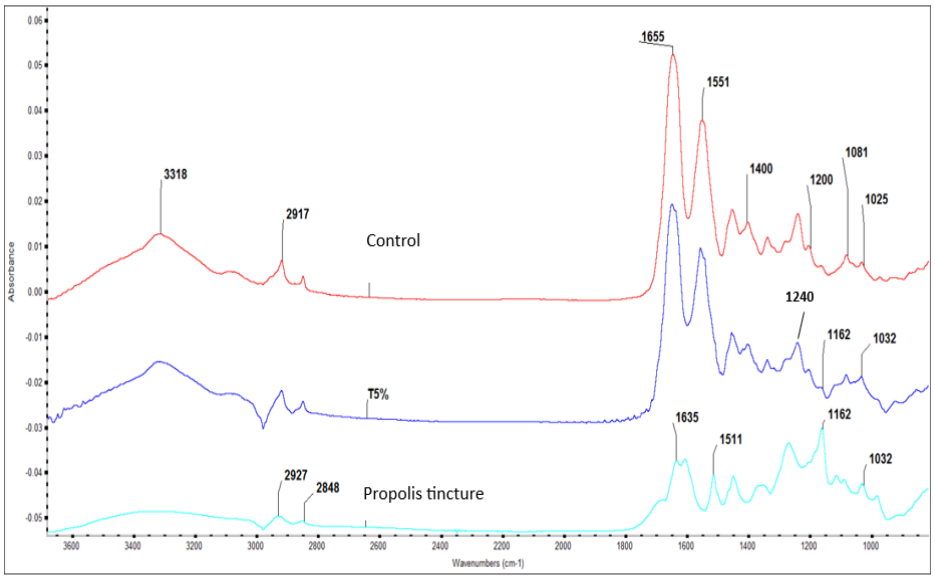

Fig. 3. FT-IR Spectra of Raw Materials (collagen-control and propolis tincture) and sample T 5%.

To illustrate the spectral changes in collagen-based materials and propolis tincture, the sample T 5% was selected for demonstration. The spectra of samples T 5%, T 3%, and T 1% are similar. In the spectrum of sample T 5%, specific collagen peaks (Amide I, Amide II, Amide III) can be observed, while the presence of propolis is highlighted by the peaks at 1162 cm^{-1} and 1032 cm^{-1}, which correspond to the aromatic rings of flavonoids.

4.2. Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was used to highlight the presence of propolis in the polymeric matrix. The DSC curve of collagen from the control sample indicates a peak at 75 °C (with an associated enthalpy of 186 J/g), which reveals an endothermic effect related to the denaturation of collagen (Fig. 4). Denaturation—distinct from degradation—involves the breaking of intercalated hydrogen bonds and leads to the formation of an amorphous polymer known as gelatin. In most sponges containing propolis tincture, the denaturation temperature of collagen is higher than that of the control sample. This suggests that the propolis tincture acts as a reinforcing agent, which may explain this effect.

The T5% sample (pink curve) shows a shift in the thermal profile compared to the control, indicating that the concentration of propolis modifies the thermal properties of the collagen matrix.

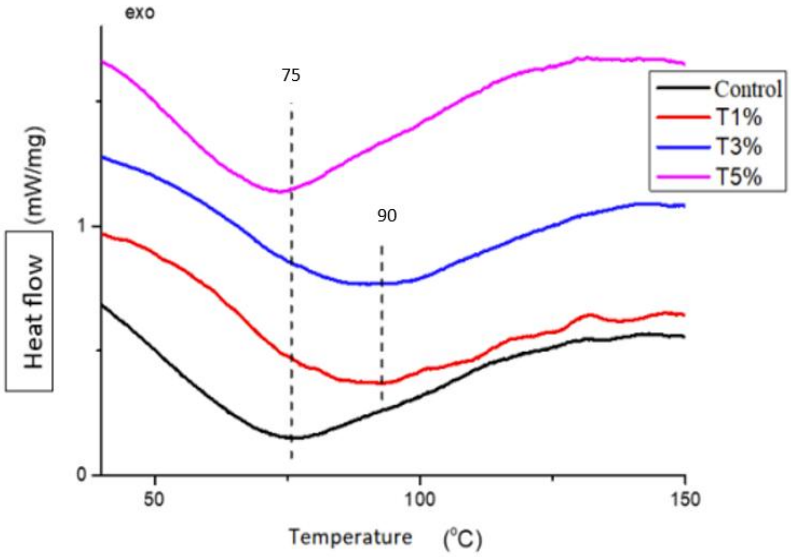

Fig. 4. DSC Curve for Control, T 1%, T 3%, and T 5% samples.

Propolis contains polyphenols and flavonoids, which can interact with collagen through hydrogen bonding and hydrophobic interactions, potentially stabilizing the structure and increasing resistance to thermal degradation. The endothermic transition (downward trend) suggests denaturation of collagen fibers, typically occurring between 60–100°C, depending on crosslinking and structural modifications.

The T5% sample requires more energy for this transition, implying stronger molecular interactions between collagen and propolis components, delaying collagen denaturation.

Some components of propolis may act as natural crosslinkers, enhancing collagen stability, which would explain why T5% exhibits a more pronounced endothermic response compared to lower concentrations (T1% and T3%).

4.3. Micro CT Analysis

Micro CT analysis was conducted to provide specific information regarding the morphology of the collagen-based samples and propolis tincture, particularly detailed data on the porosity of the investigated spongy forms. The images obtained from the Micro CT technique reveal the presence of pores in all samples, with the significant finding being that the pores are open (Fig. 5). Given that the volume of

closed pores is minimal, it does not affect the overall porosity, which is equivalent to that of the open pores.

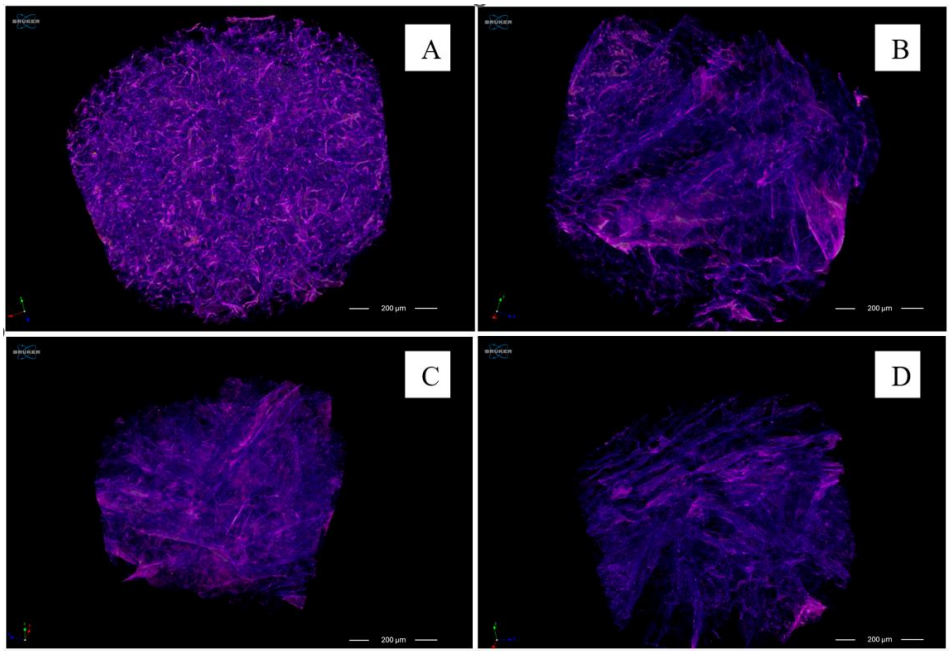

Fig. 5. Micro CT Images of Control Sample (A), T 1% (B), T 3% (C), and T 5% (D).

Regarding the material morphology, the control sample exhibits uniformly distributed open pores that are smaller in size compared to those in the samples containing propolis tincture. In the T 1%, T 3%, and T 5% samples, the total porosity is approximately 95%, with significantly larger pores, showing a total volume ranging from 1.8×10^{10} to 3.8×10^{10}. As the concentration of propolis increases, the pore connectivity density decreases, with the control sample having the highest value (3.23E -4).

4.4. Enzymatic Degradation

In vitro biodegradation of the collagen composites using collagenase solution was evaluated to simulate the *in vivo* behavior of these composites as wound dressings. According to the graph in Fig. 6, the control sample degrades the fastest, with complete degradation occurring after 4 hours. This was expected, as collagenase is an enzyme that specifically degrades collagen. The T 1% sample shows slower degradation compared to the control, but it also fully degrades after 24 hours in the collagenase solution. In contrast, the T 3% and T 5% samples exhibit partial degradation, reaching 82% and 76% degradation, respectively, after 48 hours of exposure to the enzymatic solution. The amount of propolis tincture is inversely proportional to the rate of enzymatic degradation; as the quantity of propolis

increases, the degree of degradation decreases, which is attributed to the crosslinking of collagen with propolis tincture.

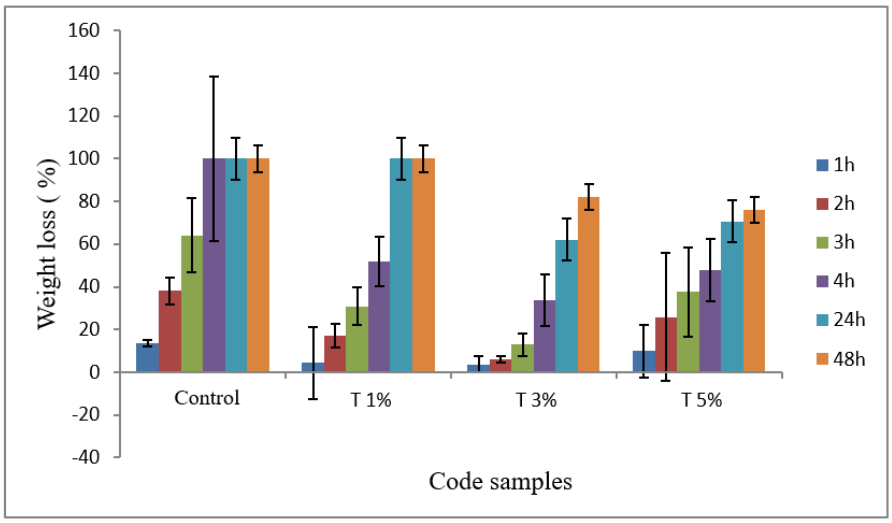

Fig. 6. Enzymatic Degradation of Collagen and Propolis Tincture Samples.

4.5. UV-VIS Analysis

The UV-VIS analysis was conducted to determine the release profile of the active substance (propolis) from the polymer matrix. According to the graph (Fig. 7), a significant release of propolis occurs within the first 24 hours.

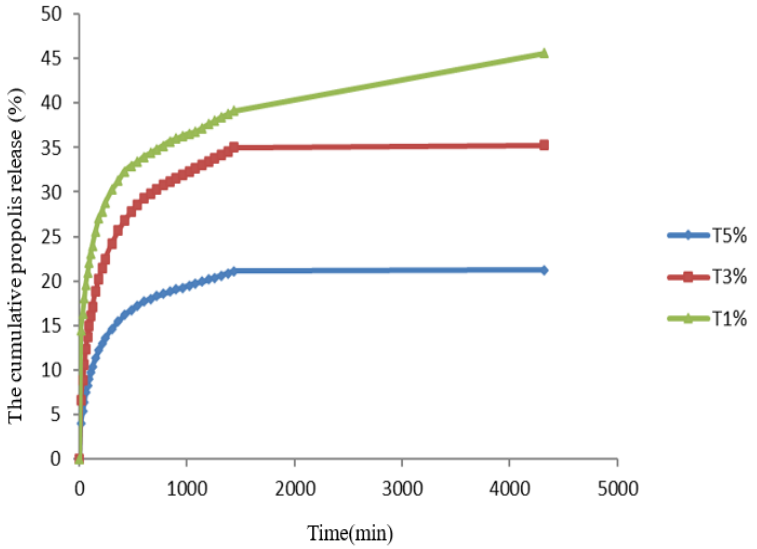

Fig. 7. Release Profiles of T 1%, T 3%, and T 5% Samples in Phosphate Buffer pH 7.2.

Analyzing the release profiles of the collagen and propolis tincture samples some intriguing insights into their behavior are revealed. The sample with the lowest concentration of propolis tincture (1%) demonstrates the highest amount of released propolis. This observation suggests that lower concentrations may facilitate a more effective release mechanism, possibly due to less obstruction within the polymer matrix.

As the concentration of the tincture increases, the kinetics of propolis release changes significantly. The 5% tincture sample shows the slowest release rate, which could be attributed to a few factors. One potential explanation is the uneven dispersion of propolis within the polymer matrix. When propolis is present in higher concentrations, it may not integrate as seamlessly, leading to localized clusters that can hinder the diffusion process.

Moreover, propolis functions as a reinforcing and filling agent within the matrix. This characteristic could further complicate the release dynamics. Essentially, as the quantity of propolis increases, the matrix may become denser, making it more challenging for the active substance to migrate out of the material.

The interconnectivity of the pores within the samples also plays a crucial role. The T 1% sample exhibits greater pore connectivity compared to the T 3% and T 5% samples. This higher connectivity allows for quicker diffusion of the active substance into the surrounding medium, facilitating a more rapid release. Conversely, in samples with lower connectivity, such as those with higher propolis concentrations, the pathway for diffusion may be obstructed, resulting in slower release rates.

Overall, these findings suggest that optimizing the concentration of propolis in the polymer matrix is critical for achieving desired release profiles. Balancing the benefits of reinforcement with the need for efficient diffusion can help tailor the properties of these biomaterials for specific applications, particularly in wound healing and tissue regeneration contexts.

5. Conclusions

The paper addressed the obtaining of systems for wound healing using natural active substances such as collagen and propolis tincture. Collagen was used as a bioactive matrix with an important role in epithelial regeneration and propolis for its antibacterial activity. The systems obtained were analyzed by FT-IR spectroscopy, DSC analysis, Micro CT, UV-VIS spectroscopy, and enzymatic degradation.

The FT-IR and DSC analyses provide compelling evidence for the integration of propolis into the collagen matrices. Specifically, the FT-IR spectrum showcases distinct bands that correspond to the aromatic rings of flavonoids, key components of propolis. This confirms not only the presence of propolis but also

its structural integration within the collagen framework. Furthermore, the DSC curve indicates that the denaturation temperature of the material is elevated in the presence of propolis, suggesting enhanced thermal stability and potential improvements in the mechanical properties of the collagen matrix due to the reinforcing effects of propolis.

In addition to the spectroscopic analyses, the Micro CT imaging reveals that all samples contain pores, with a notable emphasis on open porosity. Open pores facilitate gas exchange and nutrient diffusion, which are essential for tissue regeneration. The negligible amount of closed porosity suggests that the system is well-structured to support cellular infiltration and migration, further enhancing its suitability for dermal applications.

The enzymatic degradation tests provide valuable insights into the behavior of the collagen matrices in a biological context. As the concentration of propolis tincture increases, the rate of enzymatic degradation decreases. This inverse relationship highlights the protective role of propolis, suggesting that its incorporation into the collagen matrix may confer additional resilience against enzymatic breakdown, which is particularly beneficial in wound care scenarios where prolonged material integrity is desirable.

UV-VIS analyses reveal a critical relationship between the concentration of propolis tincture and the interconnectivity of pores.

In conclusion, the comprehensive analysis of collagen-based dressings incorporating propolis tincture illustrates the potential of this system as a biomaterial for wound treatment. Future research could focus on fine-tuning the formulation to maximize therapeutic efficacy while ensuring the system maintains its structural integrity and functionality over time.

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