The influence of propolis nanoparticles on dermal fibroblasts migration: premises for development of propolis-based collagen dermal patches

P. M. Pasca, S. Cavalu*
University of Oradea, Faculty of Medicine and Pharmacy, 10 P-ta 1 Decembrie, 410073 Oradea, Romania

Knowing the biological and pharmacological properties of propolis, the first goal of our study was to prepare and characterize a propolis nano-formulation (NPs) in order to be used for wound healing applications. The ability of propolis NPs to stimulate the migration of dermal fibroblasts in vitro was assessed by scratch test assay. The concentration of 200 μg/mL propolis NPs was found to have similar effect as the positive control. The second goal was to prepare a propolis-collagen membrane and to investigate the morphological and nanoindentation properties by AFM. The ultrastructure network of collagen fibrils was not affected by incorporation of propolis NPs, showing a nano-porous structure, favorable for soft tissue regeneration applications. Enzymatic degradation assay indicated a reduced degradation rate upon incorporation of propolis NPs in collagen matrix.

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1. Introduction

The biological and pharmacological properties of propolis formulations are well known in terms of antibacterial, antiinflamatory, antifungal, antioxidant, immunomodulatory, among others, being one of the preferred natural product to be consider for development of new drugs [1-4]. Despite the difficulties related to propolis standardization, it is generally accepted that propolis biological properties are connected to their botanical source, collection season and chemical composition [5, 6]. Romanian propolis, especially originating from Transylvania, is renowned as a high quality product, with complex chemical composition, which possess high amounts of biologically active molecules from the classes of phenolic acids and flavonoids [7, 8]. Due to its special quality, the Romanian propolis was used in different pharmaceutical and cosmetic formulations, being effective in skin's protective mechanisms and management of burns wounds [9, 10]. Propolis, as a natural bioresource, represent a reservoir of bio- compounds capable of stimulating dermal cells proliferation in addition to bearing antimicrobial or anti-inflammatory properties [10]. However, acute or chronic wounds still remains a major clinical problem, as the treatment strategy in the case of severe skin damage is to ensure rapid wound closure and re-epithelialization, providing both a moist environment and topical nutrition to promote the new tissue growth. A novel strategy, including nanoparticles and nanostructures formulations, is based on their unique physicochemical properties: ultra-small size, large surface area/mass ratio and increased chemical reactivity. The capacity of different nano-formulations with antimicrobial agents to accelerate cutaneous wound healing in vitro and in vivo was demonstrated in several papers, such as silver nanoparticles loaded collagen/chitosan scaffolds [11], or chitin/nanosilver composite scaffolds [12], exploiting the wellknown antimicrobial effect of silver nanoparticles incorporated in different natural polymeric combination.

Until present, the research studies on propolis nanoparticles usually remained limited to their effectiveness against bacterial biofilms e.g. Enterococcus faecalis biofilm in the root canal [13, 14] or adjuvant in cancer therapy to reduce the toxic side-effects caused by common chemotherapeutic agents [15]. It was also reported that antimicrobial activity of propolis nano-formulations is much more effective than conventional propolis extracts [16], while antitumor

* Corresponding author: simona.cavalu@gmail.com

investigations performed on different cells lines have revealed that the antiproliferative effects of propolis nanoparticles are much better than conventional propolis [17].

In vitro, propolis extract originated from different geographical area has been evaluated for its cytotoxicity and regenerative capacity toward stem cells derived from dental pulp [18] and fibroblasts derived from skin [19], but there is a lack of information regarding the effect of propolis nanoparticles (NPs) on epithelial cells, or connective tissue. Based upon the above findings, the main goal of our work was to evaluate in vitro the influence of propolis NPs on regulation of fibroblasts migration, in order to promote wound healing. The second objective was to prepare a collagen bandage incorporating propolis NPs, to evaluate the ultrastructural features by AFM and enzymatic degradation in a collagenase assay. According to literature, collagen-based wound dressings in the form of sheets, films or membranes have been widely used in guided tissue regeneration, as emphasized in many in vitro or in vivo studies, to cover burn wound, to treat ulcers or to sustain bone regeneration [20-23].

2. Materials and methods

2.1. Preparation and characterization of propolis NPs.

Ionotropic gelation method was applied for the preparation of propolis NPS, based on the protocol described by Gan et al. [24], with modifications. The following starting solutions were prepared: 1) Chitosan powder (medium molecular weight, deacetylation degree 85%, from Sigma-Aldrich) was dissolved in acetic acid 2% obtaining a solution with concentration of 0.2% (w/v), while the pH was adjusted to 5.0; 2) Sodium tripolyphosphate (TPP) solution (0.2%w/v) was prepared in deionized water; 3) Tween 80 (Sigma-Aldrich) solution 0.4%w/v was prepared in deionized water; 3) Commercial propolis, ethanolic extract 30% (w/v) was purchased from the local company Plant Extrakt, Cluj-Napoca, Romania. Twen 80 and chitosan solutions were first mixed under constant stirring, then propolis extract was added, followed by sonication for 10 minutes until fully incorporated. TPP solution was added drop-wise under constant stirring, the ratio chitosan/TPP being 2:1. The nanoparticles were formed spontaneously due to ionic interaction between the protonated amine groups in chitosan and the negatively charged counter-ion TPP, being stabilized by Tween 80. The mixture was centrifuged at 15000 rpm, for 30 min, until the precipitate was formed and the supernatant was discarded. After filtering and washing with distilled water, the nanoparticles were freeze-dried and the resulted powder was submitted to electron microscopy investigation by TEM (TECNAI G2 F30S-TWIN, FEI), equipped with elemental analysis system (EDX). DLS measurements (Dynamic Light Scattering- ZEN 3690 Malvern Instruments, UK) were also performed in order to confirm nanoparticles formation.

2.2. In vitro tests

The ability of propolis NPs to stimulate migration of dermal fibroblasts in vitro, was assessed according to a modified scratch assay, as described in literature [19, 25-27]. Human dermal fibroblasts NHDF (ATCC, Lonza) were seeded in a concentration of 5×10^4 cells/well in 6-well plates and incubated under 5% CO_2 atmosphere at 37°C for three days until approximately 90% confluency was reached. The culture medium was Dubelcco's modified Eagle's medium containing 10% fetal bovine serum, gentamicin 50 mg/ml and recombinant fibroblasts growth factor hFG (CC-4065) 1mg/ml. After the confluence was reached, the monolayer was scraped in a straight line using a pipette tip, the cells debris was gently removed and the wells were washed with PBS solution. The plates were marked using a marker pen at the midpoint of each row of wells, to ensure the scratches were made in the same area in each well. Two different propolis nanoparticles solutions were prepared by dissolving the weighted nano-powder in DMSO in order to obtain 100 respectively 200 μg/mL concentration of propolis NPs. The test was carried out by adding separately the propolis treatment in concentration of 100 and 200 μg/mL to each well, in triplicate. The positive control was allantoin solution 50 μg/mL, while the negative control was represented by free fibroblasts culture, with no treatment. The migration of fibroblasts was monitored by taking photographic images using an inverted light microscope (Olympus CKX41) at different time intervals (6, 12, 24, 48 h) until complete closure of a wound was achieved. The

migration of the cells was assessed by measuring the wound gap distance at fixed points in the images, using the microscope application LabSense Technology Software. The percent of the wound closure, equivalent with fibroblasts migration rate, was calculated using the formula:

$$\text{Wound Closure (\%)} = \frac{Area\ (t=0) - Area(t=6;12;24;48)}{Area\ (t=0)} \text{ x } 100 \quad (1)$$

2.3. Preparation of collagen-based bandage with propolis NPs incorporated

2 g of collagen bovine dermal type I (Sigma Aldrich) was dissolved in 100 mL glacial acetic acid solution (concentration 0.02 mol/L) and centrifuged at 4500 rpm for 30 minutes. The suspension containing propolis NPs in concentrations of 200 μg/mL was sonicated for 15 minutes and then incorporated drop by drop into the collagen solution, while stirring for 20 minutes. The mixture was poured on a flat plate (glass) and frozen at -20°C until further investigations. Collagen film without any modifications was also prepared for comparison.

2.4. AFM investigation

AFM (Atomic Force Microscopy) was applied in this study in order to evaluate the topographic details of collagen membrane with and without propolis NPs incorporated. The surface images of the collagen membranes and fibers details were obtained by using Agilent 5500 AFM (Agilent Technology, Santa Clara), at room temperature and normal humidity level (50%), working in acoustic mode, by scanning the selected area with a speed of 5.361 m/s at a resolution of 512 data points. For both imaging and indentation tests, pyramidal tips were employed. The values of Young modulus were obtained from load–displacement curves, by fitting parameters, using Oliver–Pharr method [28, 29]. There were 10 indentation sites randomly chosen on the surface of each collagen sample, reaching a maximum of 2300 nm/s depth.

2.5. Collagenase degradation test.

Five specimens from each collagen membrane specimens (with and without propolis NPs) were cut into squares (30 x 30 mm^2) and weighted. Then, the specimens were immersed in a 0.25% collagenase solution (Sigma-Aldrich) and incubated at 37° C under a flux of 5% CO_2. After different time intervals (6, 12, 24, 48 and 96 hours), the specimens were carefully removed, one by one, and allowed to completely dry in order to measure their weight. The weight of the remaining mass was expressed as percentage. The measurements were carry out in triplicate.

3. Results and discussions

3.1. Characterization of propolis NPs

The morphology of propolis NPs is evidenced in Figure 1 (a-c), along with the size distribution histogram and EDX spectrum. The TEM image evidence a regular, spherical shape of nanoparticles with sharp size distribution, showing a maximum diameter value at around 180 nm, as confirmed by the DLS analysis.

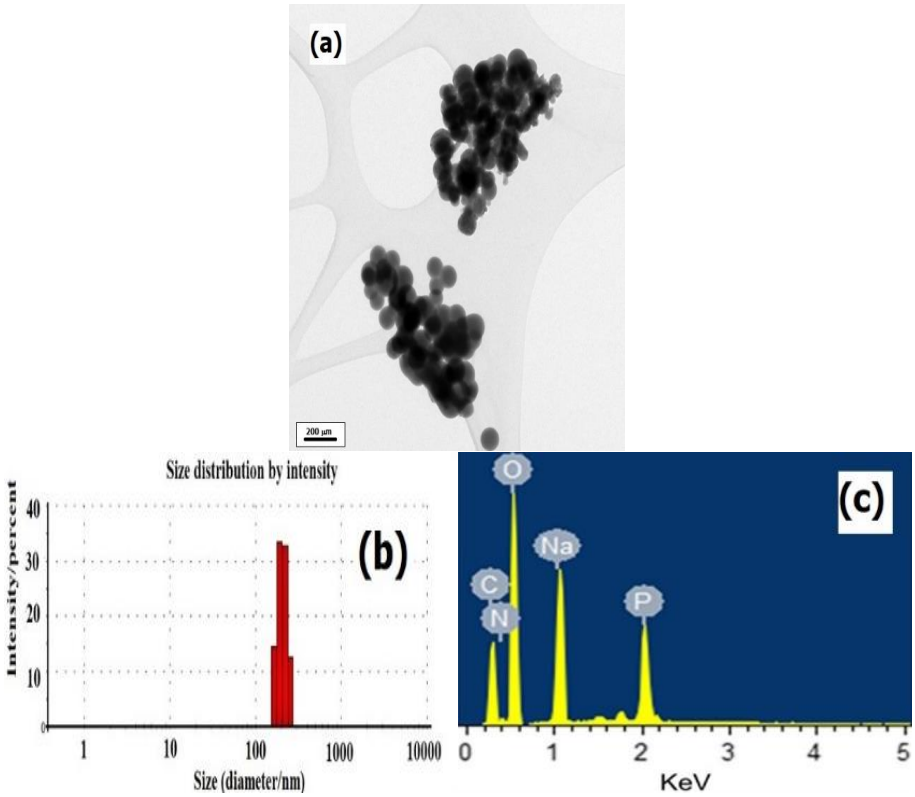

Fig. 1. (a) TEM image of propolis NPs; b) size distribution and c) EDX corresponding spectrum.

In this case, a successful propolis nano-formulation was achieved through a simple ionic gelation technique, using non-toxic chemicals, based on the ionically cross-linking between chitosan and TPP molecules (oppositely charged), entrapment of propolis extract and stabilization with Tween 80. According to literature, the combined benefits of nano-formulation and enhanced permeation properties of chitosan, are suitable for transdermal delivery of bioactive compounds [30]. So, in order to evaluate the possible application of propolis NPS for transdermal delivery, the scratch test assay was applied considering two different concentrations of propolis NPS with respect to dermal fibroblasts culture.

3.2. In vitro “scratch“ test and fibroblasts migration

The evolution of NHDF cells prior to any treatment is presented in Figure 2, showing the spontaneous proliferation in culture medium, until the confluence was achieved and formation a cellular monolayer.

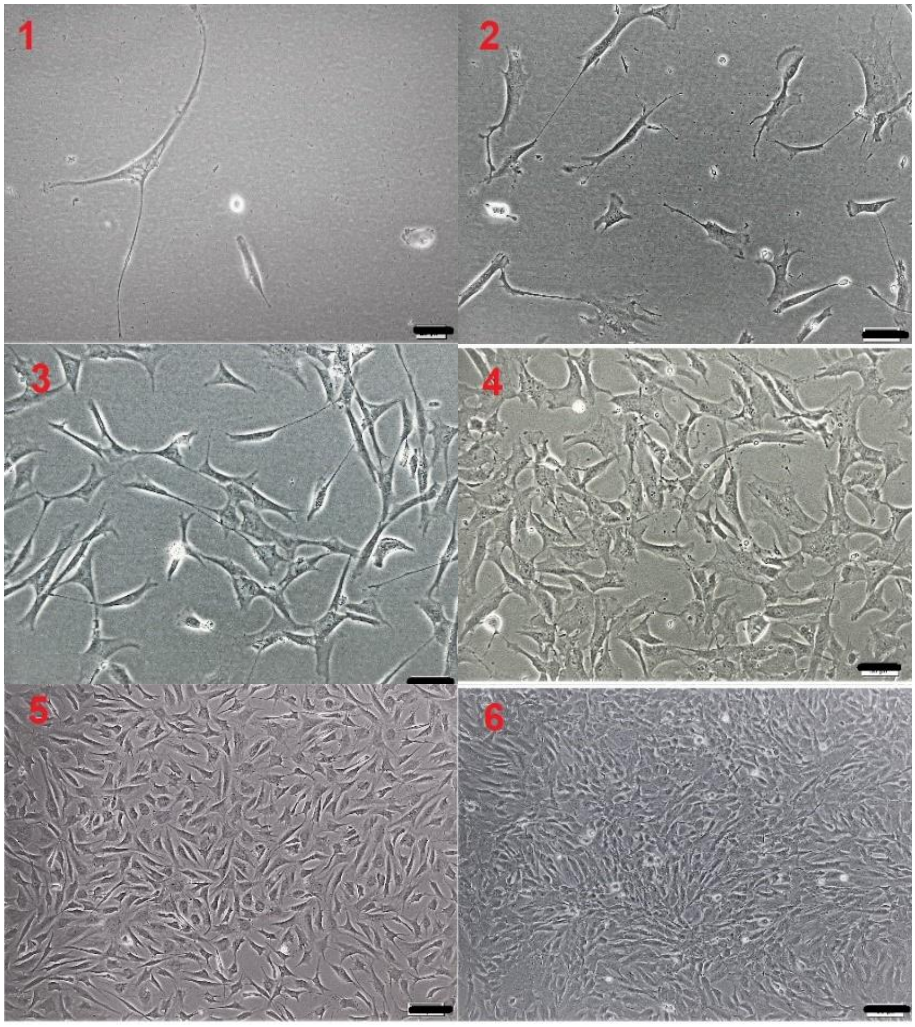

Fig. 2. Spontaneous evolution of human fibroblasts in cell culture medium, monitored at different time intervals (6, 12, 24, 48, 96 and 110 hours) until the confluence was achieved (Phase contrast image, scale bar 50 μm).

In order to assess the potential ability of propolis NPs to favor the migration of fibroblasts and the extent of regrowth to close the scratch wound, the cells culture was monitored in the presence of 100 respectively 200 μg/mL propolis NPs and compared to positive control (allantoin solution) and negative control (fibroblasts alone).

In Figure 3, the results of scratch assay are displayed, emphasizing the initial "gap" created on the fibroblasts monolayer (t=0) and the migration of the cells into the free are until the restoration of full cellular monolayer was achieved. The quantitative measurement of the wound closure expressed as percentage of the restored monolayer is presented in Figure 4.

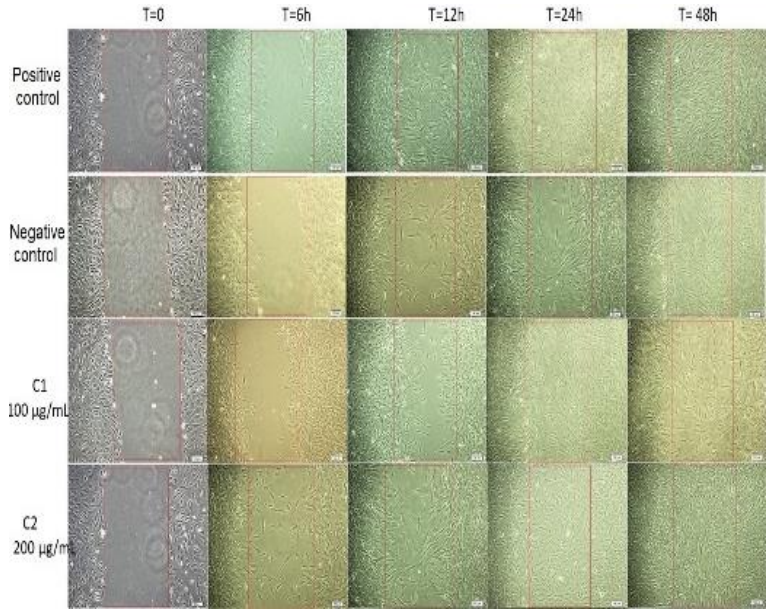

Fig. 3. Fibroblasts migration monitored after different time intervals and wound closure under the treatment with propolis NPs at two different concentrations, compared to the positive and negative control. The initial area of the scratch (t=o) is represented by the red rectangle (Phase contrast image, scale bar 100 µm).

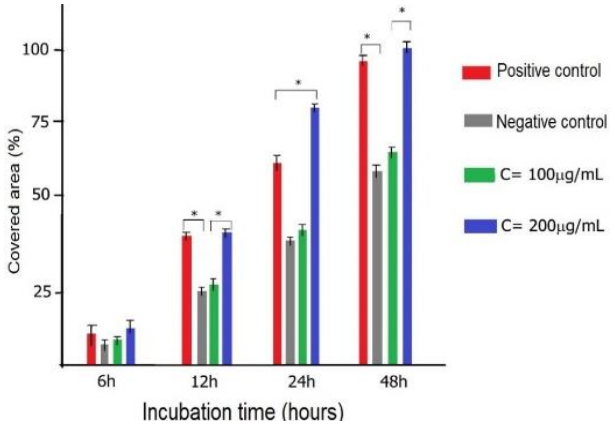

Fig. 4. The diagram representing the percent of restored fibroblasts monolayer upon migration of the cells into the free area, monitored during 48 h (Statistical relevance $p<0.05$).

One can observe that after the first 6 hours, the migration rate was very low in all the cases, regardless the concentration of NPs. After 12 hours, an increased rate of migration was noticed under the treatment with C_2 NPs concentration, similar to the positive control. The rate of fibroblasts migration was preserved after 24 hours, and the complete wound closure (100%) was achieved after 48 hours when 200 µg/mL concentration was applied, respectively 96.5 % for the positive control. By comparison, the concentration of 100 µg/mL propolis NPs resulted in 70.2 % closure, and 62.8% for the negative control, at the same point time. Moreover, after the first 24 hours, significant differences were recorded between the migration rate of fibroblastas treated with 200 µg/mL compared to the positive control, namely 82.3% towards 62.8% ($p<0.05$). Upon overall interpretation of the results we can affirm that the concentration of 200 µg/mL propolis NPs presents similar or even better effect compared to the positive control (allantoin solution), the results being promising in terms wound healing applications [31].

3.3. AFM investigation of collagen-based membrane with propolis NPs incorporated

Based on these promising results, we aimed to prepare a collagen-based bandage by incorporating propolis NPs (concentration 200 μg/mL) in collagen matrix. The topography of collagen-propolis membrane compared to neat collagen membrane is presented in Figure 5 (a-d).

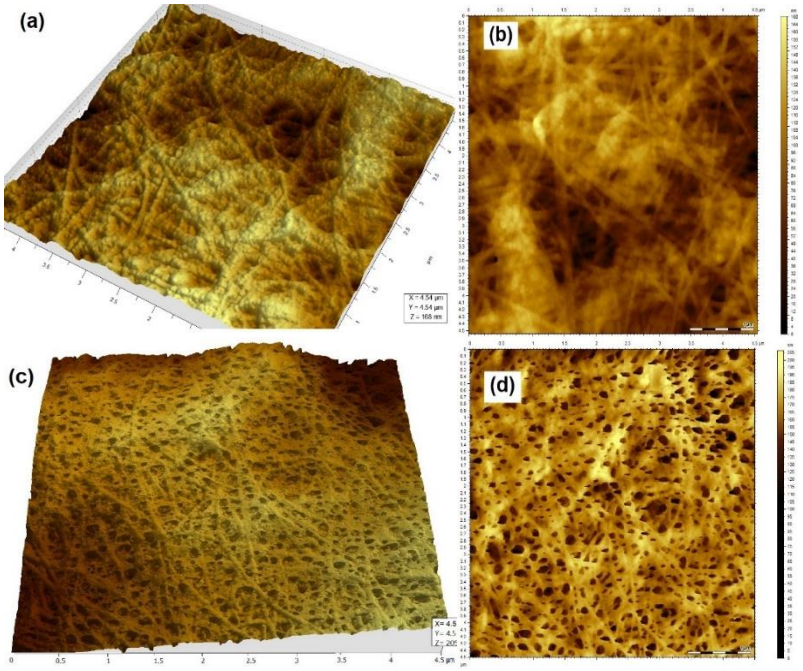

Fig. 5. AFM images of neat collagen membrane (a,b) and collagen membrane with propolis NPs incorporated (c, d), in 3D and 2D configurations.

The tridimensional network of collagen fibrils is visible in both specimens (with or without propolis NPs incorporated) emphasizing the details of repetitive structure of the D-bands pattern of a single collagen fibril, with periodic gaps and grooves, in concordance with some previous published work [32, 33]. The periodicity of D-bands is less visible after propolis NPs incorporation. Moreover, after propolis NPs incorporation and freeze drying procedure, an obvious porous ultrastructure formation was noticed, as a result of fibers self-assembly. According to recent literature, this nano-porous structure might be favorable for soft tissue regeneration applications, promoting re-epithelialization and neovascularization [20]. In order to act as a scaffold, the collagen structure should be both porous and stable to provide the diffusion of nutrients and metabolites necessary to promote wound healing. The surface topography may play an important role in cellular response to collagen. On the other hand, there is a direct influence of the scaffold architecture on its mechanical properties. The Young modulus values corresponding to neat collagen membrane and collagen with propolis NPs incorporated are presented in Figure 6, as determined by AFM nanoindentation technique.

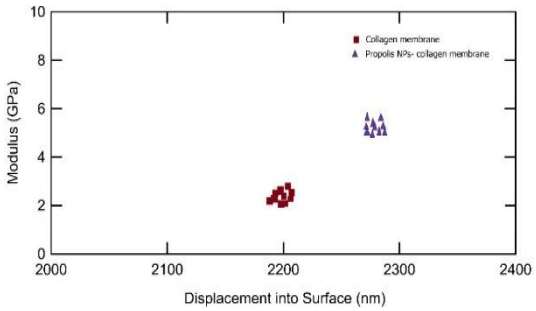

Fig. 6. The values of Young modulus coefficient obtained by AFM nanoindentation.

We noticed a slightly increase in Young modulus value of collagen membrane after incorporation of propolis NPs, from 2.8 GPa (average value of 10 measurements) to 5.7 GPa, showing good fitting results with low scattering data. These values are consistent with previous reported data on commercial collagen membranes for dental applications [29, 34], which revealed that nanoindentation measurements and the modifications of Young modulus values are correlated with the ultrastructural properties of each membrane type, especially the porosity. However, consistent changes may occur during diverse manufacturing processes to obtain convenient formulations for medical applications. Ratiu et al. demonstrated that PRGF (Plasma Rich in Growth Factors) treatment applied to commercial collagen membranes resulted in significant increase of Young modulus values, ranging in the interval 2.8–4 GPa, as determined from nanoindentation tests [29]

3.4. Enzymatic degradation assay.

The results of enzymatic degradation test using collagenase is presented in Figure 7.

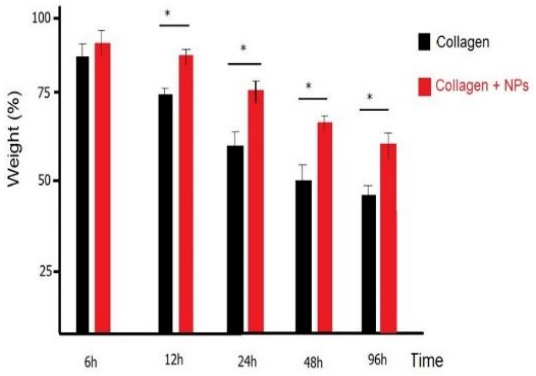

Fig. 7. Collagenase degradation test of neat collagen membrane and collagen-propolis NPs membrane (statistical relevance $p<0.05$)

As can be observed from the diagram, within the first 6 hours only a small degradation was noticed for both specimens. After 12 hours, an average percent of 25.4 % weight lost was noticed for neat collagen membrane, and 15.7% for collagen with propolis NPs, followed by similar trend in the next 48 hours, so at the final of the monitored time interval (96 h), the average weight lost was 52.1% respectively 49.8% for the collagen and collagen-propolis membrane. Overall, one can notice a reduced degradation rate upon incorporation of propolis NPs in collagen matrix. The results indicates that propolis nanoparticles may have a positive effect on the interaction between the collagen alpha-helical structures, with consequence in polypeptide chain mobility. It is well known that the major drawback of native collagen formulations is rapid degradation by enzymes (collagenase, trypsin). For tissue regeneration applications, the collagen membrane must remain chemically stable for an adequate period, in order to promote vascular endothelial cells to growth [35]. The disaggregation of collagen fibrils occurs as a consequence of the degradation of proteoglycan bridges between collagen fibrils. Our results indicates that propolis NPs exhibits a strengthening effect on these bridges, preventing a rapid degradation.

4. Conclusions

In the present study, we demonstrated that propolis NPs prepared by ionic gelation method promotes the migration and proliferation of human fibroblasts, in vitro, in a scratch test assay. The concentration of 200 μg/mL propolis NPs was found to have similar effect as the positive control (allantoin), the results being promising in terms of wound healing applications. A collagen-based membrane was prepared and investigated by AFM in terms of morphological features and nanoindentation. The network of collagen fibrils was not affected by propolis NPs, showing a

nano-porous structure, favorable for soft tissue regeneration applications. Enzymatic degradation assay indicated a reduced degradation rate upon incorporation of propolis NPs in collagen matrix. Corroborating the above mentioned results, we consider that modified-collagen membrane by adding propolis NPs in a controlled concentration, might represent a promising natural alternative to synthetic bandages for wound healing applications. Of course, further in vitro and in vivo tests are necessary to evaluate the biological performances of collagen-modified membranes, in terms of tissue adaptation and integration.

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