BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902023e201090

Article

Development and clinical application of hydrogel formulations containing papain and urea for wound healing

Thais Menezes dos Santos[1], Danieli Silva Feijó de Sousa[1], Karina Chamma Di Piero[2], Adriane Regina Todeschini[3], Wagner Barbosa Dias[3], Catarina Amorim Oliveira[4], Elisabete Pereira dos Santos[1], Mariana Sato de Souza Bustamante Monteiro[1], Maria Kátia Gomes[5], Cristiano dos Reis Moura[6], Pedro Antônio Castelo Teixeira[7], Eduardo Ricci-Junior[1]*, Zaida Maria Faria de Freitas[1]

[1]Laboratório de Desenvolvimento Galênico - LADEG, Departamento de Fármacos e Medicamentos, Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, [2]Pós-Graduação em Clínica Médica, Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, [2]Comissão de Métodos Relacionados à Integridade da Pele (COMEIP), Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, [3]Laboratório de Glicobiologia Estrutural e Funcional, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, [4]Complexo de Alimentos, Instituto Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, [5]Departamento de Medicina em Atenção Primária à Saúde e Serviço de Dermatologia, Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, [6]Agência Nacional de Saúde Suplementar, Rio de Janeiro, RJ, Brasil, [7]Laboratório de Apoio ao Desenvolvimento Tecnológico/Laboratório Brasileiro de Controle de Dopagem, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Hydrogels are used for wound treatment, as they may contain one or more active components and protect the wound bed. Papain is one of the active substances that have been used with this purpose, alongside urea. In this paper, carboxypolymethylene hydrogels containing papain (2% and 10% concentrations) and urea (5% concentration) were produced. Physical-chemical stability was performed at 0, 7, 15 and 30 days at 2-8ºC, 25ºC and 40ºC, as well as the rheological aspects and proteolytic activity of papain by gel electrophoresis. Clinical efficacy of the formulations in patients with lower limb ulcers was also evaluated in a prospective, single-center, randomized, double-blind and comparative clinical trial. The results showed 7-day stability for the formulations under 25ºC, in addition to approximately 100% and 15% of protein activity for 10% and 2% papain hydrogel, respectively. The rheological profile was non-Newtonian for the 10% papain hydrogel tested. There were no significant differences regarding the mean time for healing of the lesions, although 10% papain presented a better approach to be used in all types of tissue present in the wound bed.

Keywords: Wound healing. Hydrogel. Papain. Urea. Electrophoresis.

INTRODUCTION

Wounds consist of any interruption in continuity of the skin that affects its integrity. They can be caused by several factors, such as surgical, traumatic and ulcerative (Mandelbaum, Di Santis, Mandelbaum, 2003). For their healing, homeostasis must be regulated after

***Correspondence:** E. Ricci-Júnior. Laboratório de Desenvolvimento Galênico. Departamento de Fármacos e Medicamentos. Faculdade de Farmácia. Universidade Federal do Rio de Janeiro. Centro de Ciências da Saúde. Bloco L, Cidade Universitária, Ilha do Fundão. CP 68525, CEP: 21945-970, Rio de Janeiro, RJ, Brazil. E-mail ricci@pharma.ufrj.br. Orcid: https://orcid.org/0000-0002-2550-696X

the damage and onset of local clotting. Subsequently, the inflammatory process begins and, subsequently, cell proliferation and remodeling of the affected area (Zhao *et al*., 2016). Wounds can be classified as acute or chronic, the latter being characterized by the period necessary for their healing equal to or greater than three months (Tricco *et al*., 2015). For this reason, they are considered a public health problem, as delays in the healing process affect rehabilitation of the patient and increase the care costs (Oliveira, Castro, Granjeiro, 2013). Chronic wounds are often caused by complications of diabetes, vascular ulcers and pressure ulcers (Zhao *et al*., 2011; Das, Baker, 2016; Larouche *et al*., 2018; Okur *et al*., 2020). In particular, lower limb ulcers have high incidence and prevalence, with venous ulcers as one of their main causes and an incidence rate of up to 80.0%, with the possibility of affecting from young to aged individuals (Abbade, Lastória, Almeida, 2011).

Currently, there are different treatments for wounds, which vary according to the tissue characteristics. Generally, the criteria used to choose the best treatment take the following into account: its ability to maintain the wound bed moist; the need to treat bacterial bioburden; wound exudate nature and volume; condition of the tissue in the ulcer bed; perilesional skin condition; ulcer size, depth and location; presence of tunneling and/or cavitation; and care of the individual with an ulcer (National Pressure Ulcer Advisory Panel, 2014).

Among the possible topical treatments, the following stand out: semi-permeable films; semi-permeable, non-adherent, hydrocolloid alginate; hydrofiber; foam and antimicrobial dressings; hydrogels; larval therapy and modulating matrix with proteases, in addition to complementary therapies such as negative pressure therapy, laser therapy, oxygen therapy and hyperbaric therapy, among others. Availability of these products, their cost, and the patient's access determine the most appropriate course of action to be used in the treatment (Abdelrahman, Newton, 2011).

Hydrogels are a very promising alternative, as they may contain one or more active components, can retain water in their structure, present low penetrating power in the skin, assist in the local action of autolytic debridement and maintain the environment moist, aiding wound healing. In addition, they are easily distributed over the lesions without exceeding their limits, with easy removal and without leaving residues (Ahmed, 2015; Varaprasad *et al*., 2017). Viscous hydrogels are also able to promote wound filling, contributing to a remodeling phase (Akash, Rehman, 2015; Das *et al*., 2015; Gainza *et al*., 2015; Powers *et al*., 2016; Silva *et al*., 2015). Therefore, formulations containing active substances and formulated using hydrogels tend to provide treatments with more promising results.

Papain is one of the active substances that have been used in recent decades as an aid in healing processes. It is a proteolytic enzyme that originates from papaya latex (*Carica papaya*), commonly found in Brazil; It has low cost, reduced adverse effects, selective enzymatic debridement action, and bactericidal and bacteriostatic potentials, contributing in all phases of the healing process of acute and chronic wounds (Lantis, Paredes, 2017). It is classified as a protease cysteine and has a molecular weight of 23.4 kDa and 212 residues (Fernández-Lucas, Castañeda, Hormigo, 2017). According to the Kyoto Encyclopedia of Genes and Genomes – KEGG and Biochemical Nomenclature Committee IUBMB/IUPAC (KEGG, 2020), papain has identification number 3.4.22.2. This means that it belongs to the group of hydrolases (3), peptidases (4), and cysteine endopeptidases (22). The papain molecule has a sulfhydryl radical (-SH) in its active site, specifically in the cysteine residue, being essential that this group is free for the action of the enzymatic activity in its reduced form.

Papain can be used without any association with other substances or associated with urea. The function of urea is to facilitate the proteolytic action of papain, as it alters the enzyme's three-dimensional structure, breaking its hydrogen bonds and exposing the cysteine residues of its active sites. This combination also allows for non-specific debridement, within a wide pH range (3.0 - 12.0), thus providing greater stability of the drug (Goldman, 1959).

Topical formulations are effective therapeutic alternatives, offering low cost, low technological complexity, few adverse effects, and easy access for patients. In Brazil, commercialization of papain hydrogels is usually through compounding pharmacies, based on the

Brazilian Pharmacopoeia and its National Drug Formulary (*Formulário Nacional*, 2012). Therefore, the objective of this paper was to develop two hydrogels for the treatment of wounds, containing 5% urea and papain at different concentrations: HGP2 with 2% papain, and HGP10 with 10% papain. The papain and urea concentrations followed Goldman (1959) and the National Drug Formulary (2012). The hydrogels were evaluated for physical-chemical stability at 0, 7, 15 and 30 days at 2-8ºC, 25ºC and 40ºC, as well as the rheological aspects, and proteolytic activity of papain by gel electrophoresis using bovine serum albumin (BSA) as substrate. Clinical efficacy of the formulations in patients with lower limb ulcer, treated at the Clementino Fraga Filho University Hospital (*Hospital Universitário Clementino Fraga Filho*, HUCFF), Rio de Janeiro, Brazil, was also evaluated in a prospective, single-center, randomized, double-blind and comparative clinical trial, applying the PUSH instrument, version 3.0, to assess evolution of wound healing.

MATERIAL AND METHODS

Material

The reference chemical substance (RCS) was papain purchased from Sigma-Aldrich Brasil Ltda (Rio de Janeiro, RJ); whereas urea was purchased from Infinity Pharma (Campinas, SP). The excipients used in preparing the formulation were as follows: Carboxypolymethylene (CPM) purchased from Galena (Campinas, SP); Aminomethylpropanol (AMP-95) purchased from Pharma Nostra (Campinas, SP); Glycerin purchased from Henrifarma (Cambuci, SP); Solution of Phenoxyethanol and Parabens and Urea purchased from Fagron (São Paulo, SP); and Sodium borate purchased from Farmos (Rio de Janeiro, RJ).

Preparation of hydrogels with papain and urea

Table I shows the composition of the HGP2 and HGP10 formulations, which were prepared by means of the classic method. Distilled water, glycerin and a solution of phenoxyethanol and parabens were mixed in a stainless steel container. Subsequently, CPM was added and stirred until complete dispersion. AMP-95 was then added and the system was kept under mechanical stirring until complete homogenization. Papain, urea and sodium borate were pulverized and levigated with glycerin forming a mixture of a pasty consistency. Subsequently, this mixture was placed in a stainless steel container and the previously prepared hydrogel was gradually transferred under mechanical stirring, until complete homogenization. Hydrogels without papain and without urea, called HGWA, were also developed. A formulation called HGSPU was also produced as a control: it is a formulation frequently used by patients at the University Pharmacy of the Federal University of Rio de Janeiro (where all the formulations were developed) for wound treatment, containing 10% papain but without urea or sodium borate. The hydrogels were produced according to the Good Practices described in Collegiate Board Resolution No. 67/2007, which only foresees sterilization for magistral parental and ocular products (ANVISA, 2007).

TABLE I - Hydrogels components, with urea and papain – HGP2 and HGP10

Components	Percentage in the formulations (% p/p)			
	HGP2	HGP10	HGWA	HGSPU
Carboxypolymethylene	2.0	2.0	2.0	2.0
Papain	2.0	10,0	-	10.0
Urea	5.0	5.0	-	
*Phenoxyethanol + Paraben blend	0.3	0.3	0.3	0.3
Sodium borate	1.0	1.0	1.0	-

TABLE I - Hydrogels components, with urea and papain – HGP2 and HGP10

Components	Percentage in the formulations (% p/p)			
	HGP2	HGP10	HGWA	HGSPU
Glycerin	20.0	20.0	20.0	20.0
Distilled water	qs 100.0			

*Phenoxyethanol + Paraben blend: methylparaben, ethylparaben, propylparaben, butylparaben and isobutylparaben.

Hydrogel characterization, stability tests and organoleptic aspects

HGP2, HGP10 and HGWA were submitted to the stability test for 30 days at 25 ± 2ºC and at 40 ± 2ºC, for 0, 7, 15 and 30 days.

The samples were evaluated in triplicate (n = 3) for organoleptic aspects, pH measurements, density and proteolytic activity (ANVISA, 2005). Proteolytic activity of the HGP10 hydrogel at 2-8º°C was also performed, being compared with the hydrogel without urea and with papain (called HGSU) and with the hydrogel without papain or urea (HGWA), from 0 days to 60 days.

The macroscopic evaluation was carried out by analyzing the color, physical aspect and state of the matter of the formulations (Buchmann, 2001).

The pH values of the hydrogels were determined by means of a potentiometric method at 25ºC, using an MS Tecnopon potentiometer, Model mPA210. The mean ± standard deviation was evaluated.

Density (d) was evaluated using a Hubbard pycnometer (25 mL). The empty pycnometer was weighed on an analytical scale (p1). Subsequently, it was filled with distilled water and weighted (p2). The same procedure was performed with the hydrogel samples added to the pycnometer (p3), avoiding formation of bubbles (Brazilian Pharmacopeia, 2019). This procedure was performed in triplicate (n = 3). Equation 1 below was used to assess density.

$$d = p3 - p2/p2 - p1 \quad \text{(Equation 1)}$$

Rheological characterization

An Anton Paar MCR 302 rheometer with the Rheoplus/32 software was used for rheological measurements of the HGP2, HGP10 and HGWA samples. PP-20 (parallel plate with 20 mm diameter) and CP-40-2 (cone-plate with 40 mm diameter and angle of 2º) type geometry were used during the rotational and oscillatory analyses, respectively. Viscosity and flow curves of the hydrogels were obtained by applying a shear rate in the range from 1 to 300 s^{-1} at 5, 20 and 32°C in CSR (*Controlled Shear Rate*) mode (Grip *et al*., 2017)[28].

Amplitude sweeps (constant frequency of 10 $rad.s^{-1}$) were also carried out to obtain the structural characteristics of the samples without HGWA and with 10% w/w active HGP10 at 20°C. The storage G′ and loss G″ moduli were examined as a function of strain, as well as the region of linear viscoelasticity (LVE) and delta tangent (tanδ) (Oliveira *et al*., 2018).

Analytical validation of the electrophoresis method

The method was validated using the Polyacrylamide Gel Electrophoresis (PAGE) technique with surfactant Dodecyl Sulfate Sodium (DSS) and Bovine Serum Albumin (BSA), as substrate. The parameters evaluated in the validation were the following: selectivity, linearity, accuracy, precision, limit of detection (LoD), limit of quantitation (LoQ), and robustness (Brunelle, Green, 2014; Reichel, Thevis, 2013). For this purpose, the test evaluated the proteolytic activity of papain present in the formulations, at different temperatures (2-8ºC, 25ºC, and 40ºC).

Linearity of the method was evaluated from the result of five standard curves (n = 5 determinations) relating the enzymatic activity of papain (densitometry) to a standard BSA curve (from 0.0125 to 0.20 mg of BSA, from a 20 mg/mL stock solution). ANOVA analysis was used for data analysis, with a 5% ($\alpha = 0.05$) significance level. An F-Statistics test was also performed, which assesses the ratio of the variance between the data series and the variance between the data in a data series. As null hypothesis (H_o), it was considered that the values obtained for the data series mean are not different from each other and, consequently, the method is linear; as an alternative hypothesis (H_1), it was considered that the values differ from each other and, consequently, the method is not linear. The correlation coefficient (r) was also evaluated, which must be above 0.990 as the minimum acceptable criteria.

Intra-day precision (repeatability) was determined from the densitometry observed for BSA (0.05 mg, 0.10 mg and 0.20 mg) in triplicate (n = 3), totaling 9 determinations. In the evaluation of inter-day precision (intermediate precision), densitometry was evaluated for the same BSA curve in triplicate (n = 3) and by a second analyst, totaling 18 determinations. Subsequently, the relative Standard Deviation (SD) was calculated, according to Equation 2.

$$SD = \frac{D}{MC} X\ 100 \quad \text{(Equation 2)}$$

D corresponds to the standard deviation and MC is the mean concentration. A DPR value below 5.0% was used as the minimum acceptable criterion.

Accuracy was determined by recovering the analyte in samples containing known amounts of BSA, totaling 9 determinations. For this purpose, densitometry was determined from 0.05 mg, 0.10 mg and 0.20 mg of BSA, simulating consumption of the analyte after presence of papain during the sample tests. Recovery (%) was calculated using Equation 3:

$$\text{Recovery} = \frac{\text{Experimental mean concentration}}{\text{Therorical concentration}} \times 100 \quad \text{(Equation 3)}$$

The acceptance criteria for recovery were as follows: objective of the method, intrinsic variability of the method, work concentration, and concentration of the analyte in the sample (ANVISA, 2017).

Quantitative evaluation of papain by means of electrophoresis

The quantitative evaluation of the proteolytic activity of papain in the HGP2 and HGP10 formulations was performed using BSA as substrate. In triplicate, from 0.006 g to 0.0140 g of the gel were weighed and subsequently diluted one hundred times in a buffer (50 mM Tris pH6.8; 5 Mm Cysteine, 1 mM EDTA). The diluted sample (90 µl) and 10 µl of BSA (20 mg/mL) were stirred in a 37°C water bath. After 1 hour, 20 µl of β-mercaptoethanol buffer were added and heated in a dry bath at 100°C for 5 minutes. The proteins were separated on a gel of 12% Dodecyl Sodium Sulfate Polyacrylamide (SDS-PAGE) at 120 V and 3.00 A and stained in an 80% Coomassie Blue solution and 20% Methanol for 24 hours. Protein quantification was performed by means of ImageJ - Image Processing and Analysis in Java after gel discoloration. The study was carried out in triplicate. The calibration curve was prepared using the density of the bands versus the volume of the substrate applied to the wells; concentration of the sample was obtained from the linear regression of this calibration curve, (Thierry, Maillard, Lortal, 2002; Yamamoto, Yamaguchi, Nagamune, 2008).

Evaluation of clinical efficacy

The study was approved by the Research Ethics Committee of the Clementino Fraga Filho University Hospital (HUCFF) belonging to the Federal University of Rio de Janeiro (*Universidade Federal de Rio de Janeiro*, UFRJ), Brazil, protocol No. 78755617.6.0000.5257-2018, and conducted according to the methodology described by Schulz & Altman for the CONSORT Group (Schulz, Altman, Moher, 2010).

A clinical, prospective, single-center, randomized, double-blind and comparative trial was conducted with 62 patients with lower limb ulcers from HUCFF. They were divided into two groups and treated with the HGP2 (32 patients) and HGP10 (30 patients) formulations, in order to compare effectiveness of the formulations, evaluating

the healing time of the lesions (Schulz, Altman, Moher, 2010)]. The groups had volunteers with different profiles: both gender; different skin color/race; aged between 35 and 91 years old; and injury time from 25 days to 10 years. There were three possible causes of injuries: chronic venous insufficiency, neuropathy secondary to diabetes mellitus, and Hansen's disease.

Using the Pressure Ulcer Scale for Healing (PUSH), adapted version 3.0, the wound healing performance of all patients was assessed weekly. The instrument employs three variables: wound area (length and width), amount of exudate present in the wound, and appearance of the wound bed (the type of tissue prevalent in this region - slough, coagulation necrosis, granulation and epithelialization) for a systematic assessment of the healing evolution capable of generating greater safety in continuity of the therapy. Specific subscores were assigned to each parameter evaluated. When added up, these variables yielded a score ranging from 0 to 17, where higher values indicate worse patient prognosis and score zero indicates good healing prognosis with a cure (Santos *et al*., 2005).

Statistical analysis

The experimental results obtained were expressed as mean ± standard deviation. The results were submitted to statistical analysis using the GraphPad Prism® software, version 5.0 for Windows. The statistical analysis of variance test (ANOVA) was carried out, with statistical significance set at 5% ($p<0.05$), for group analysis. For paired analysis, the t-test was used with statistical significance set at 5% ($p<0.05$).

RESULTS

Preparation, characterizations and stability tests

The formulations developed showed a stable and homogeneous appearance. However, they were stored away from oxidizing materials and agents, such as oxygen, oxygen water, light and heat, to reduce oxidation of the sulfhydryl radical, found in the active enzyme site (Sankalia *et al*., 2005).

Stability of the HGP2, HGP10 and HGWA formulations were analyzed for 30 days at 25°C ± 2ºC and at 40°C ± 2ºC. Organoleptic aspects, pH measurements, density and proteolytic activity were evaluated, comparing characteristics and values obtained at T0 to those obtained at times T7, T15 and T30. An analysis of the proteolytic activity of hydrogel HGP10 at 2-8°C was also performed, comparing it to the hydrogel previously used for the treatment of patients (without urea) called HGSU, from 0 days to 60 days.

The HGWA, HGP2 and HGP10 formulations showed semisolid and translucent appearance, characteristics of polymeric hydrogels, at times T0, T7, T15 and T30 and at 25ºC. However, this aspect became yellowish after 30 days at 40ºC, when compared to the other moments. This was possibly due to some degradation of papain, which is a yellowish powder.

Table II shows the results of the pH values (mean ± SD). In all three formulations, there was a statistical difference ($p<0.05$) between the time of analysis (T0, T7, T15, and T30) and temperature (25ºC and 40ºC).

TABLE II - pH values at T0 and T30 for HGWA, HGP2 and HGP10, at 25ºC and 40ºC. (*) statistical difference between T0 and the data evidenced and (**) statistical difference between the data evidenced

Time/Temperature	HGWA	HGP2	HGP10
T0 / 25ºC	4.5 ± 0.2	5.6 ± 0.1	5.5 ± 0.1
T7 / 25ºC	4.1 ± 0.1*	5.5 ± 0.1	5.4 ± 0.1
T7 /40ºC	4.4 ± 0.2	5.6 ± 0.1	5.4 ± 0.1
T15 /25ºC	4.8 ± 0.1*	5.4 ± 0.1*	5.4 ± 0.1
T15 / 40ºC	4.9 ± 0.1*	6.3 ± 0.1*	5.8 ± 0.1*
T30 / 25ºC	4.1 ± 0.1**	5.5 ± 0.1	5.2 ± 0.1*
T30 / 40ºC	4.8 ± 0.5**	5.8 ± 0.1*	5.4 ± 0.1

Table III shows the density results for all three formulations. There was a statistical difference ($p<0.05$) between time and temperatures of the same hydrogel.

TABLE III - Density values of the stability test at T0 to T30 for HGWA, HGP2 and HGP10

Time / Temperature	HGWA	HGP2	HGP10
T0 / 25ºC	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.0
T7 / 25ºC	1.0 ± 0.1	1.1 ± 0.0*	1.1 ± 0.0*
T7 /40ºC	1.0 ± 0.1	1.1 ± 0.0	1.1 ± 0.0*
T15 /25ºC	1.1 ± 0.0*	1.0 ± 0.0*	1.1 ± 0.0*
T15 / 40ºC	1.1 ± 0.0*	1.1 ± 0.0*	1.1 ± 0.0
T30 / 25ºC	0.9 ± 0.0*	1.1 ± 0.0	1.1 ± 0.0
T30 / 40ºC	0.9 ± 0.0*	1.0 ± 0.0*	1.1 ± 0.0*

Rheological characterization

Figure 1 shows the flow and viscosity curves of the hydrogels.

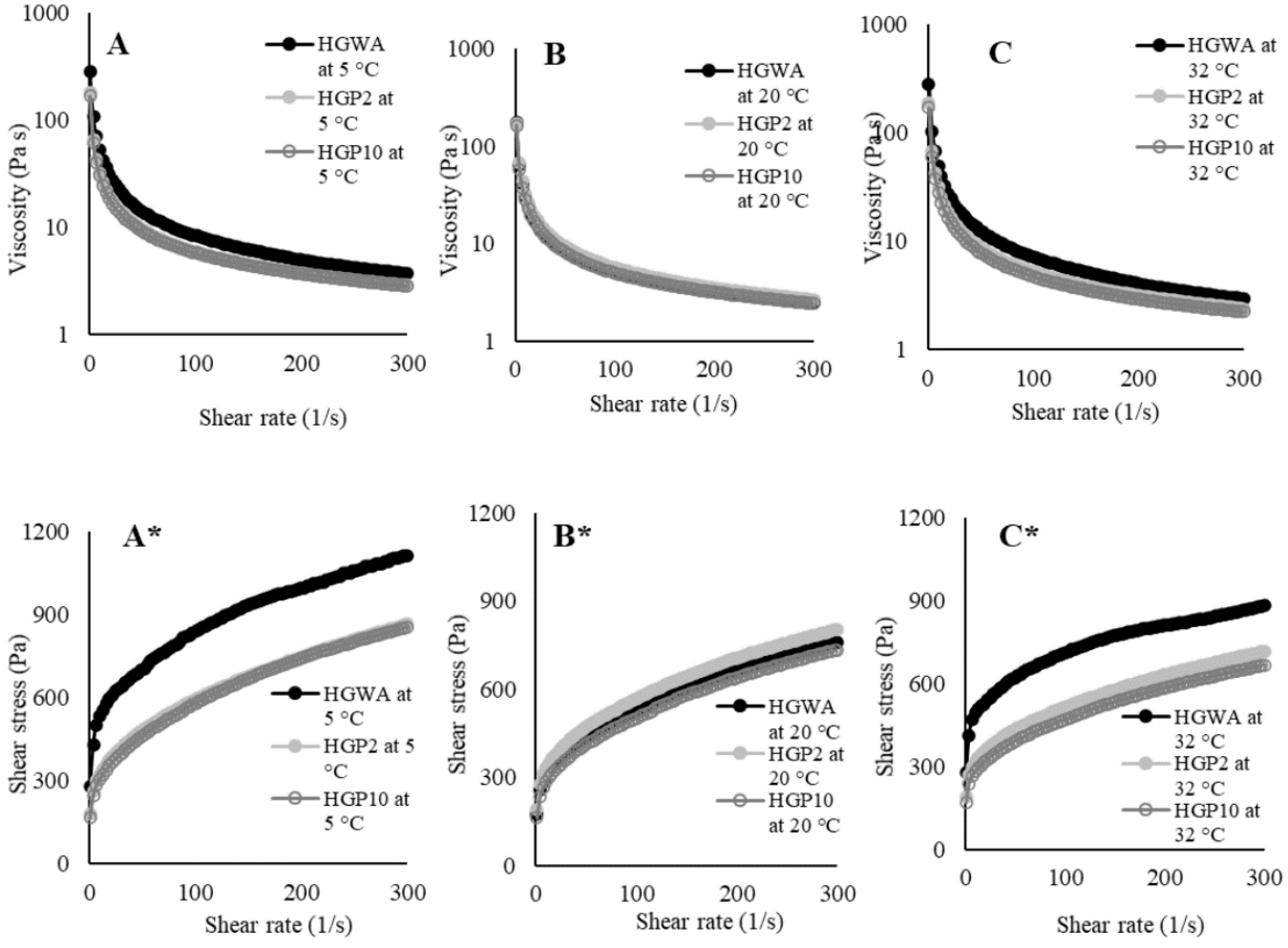

FIGURE 1 - Rheograms of the HGWA, HGP2 and HGP10 samples: Viscosity curves A, B, and C at 5°C, 20°C, and 32°C, respectively; Flow curves A*, B* and C* at 5°C, 20°C and 32°C, respectively.

Figure 2 shows the results of the oscillatory test of the hydrogels. The curves of the storage G' and loss G'' moduli of the hydrogels were successfully obtained.

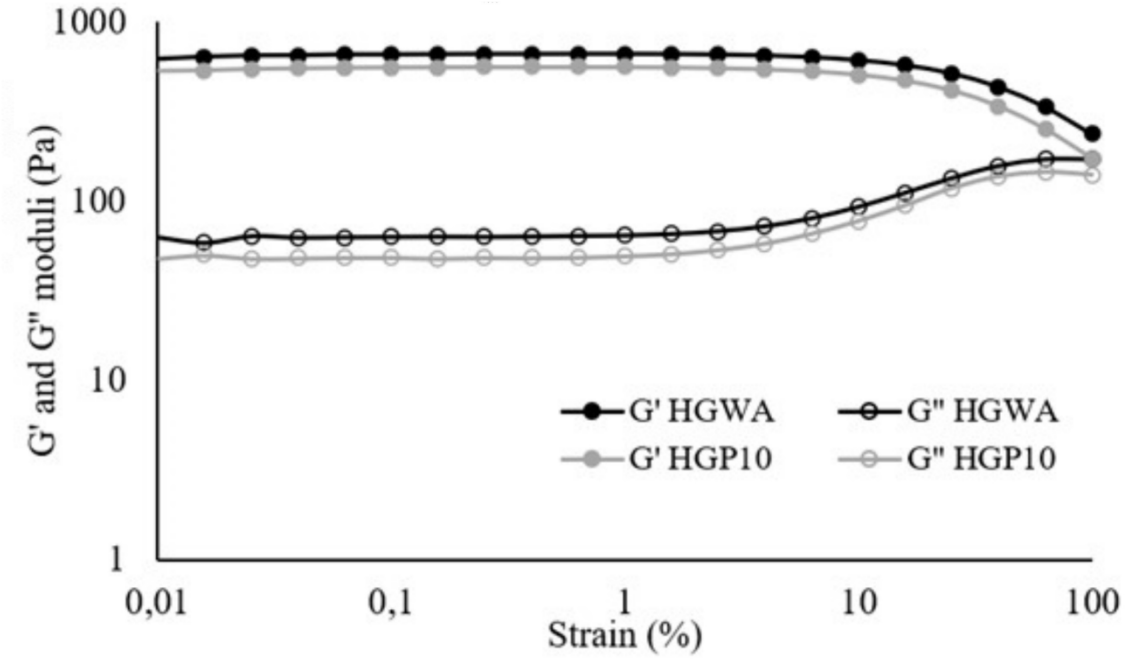

FIGURE 2 - Results of the oscillatory test of the strain sweep for hydrogels without (HGWA) and with (HGP10) active component.

Table IV shows the tanδ (G''/G') value that measures the elasticity degree of the hydrogels.

TABLE IV - tanδ[a] and LVE region limit for the HGWA and HGP10 formulations

Formulation	Tan δa	LVE region limit
HGWA	9.63 e^{-2}	γ_L = 5% $\square_y$ = 32.2 Pa
HGP10	8.71 e^{-2}	γ_L = 1% $\square_y$ = 5.63 Pa

[a] Calculated between the G''/G' ratio at 0.1% deformation

Papain proteolytic activity evaluation

Figure 3 shows the BSA calibration curves. BSA was used as papain subtract.

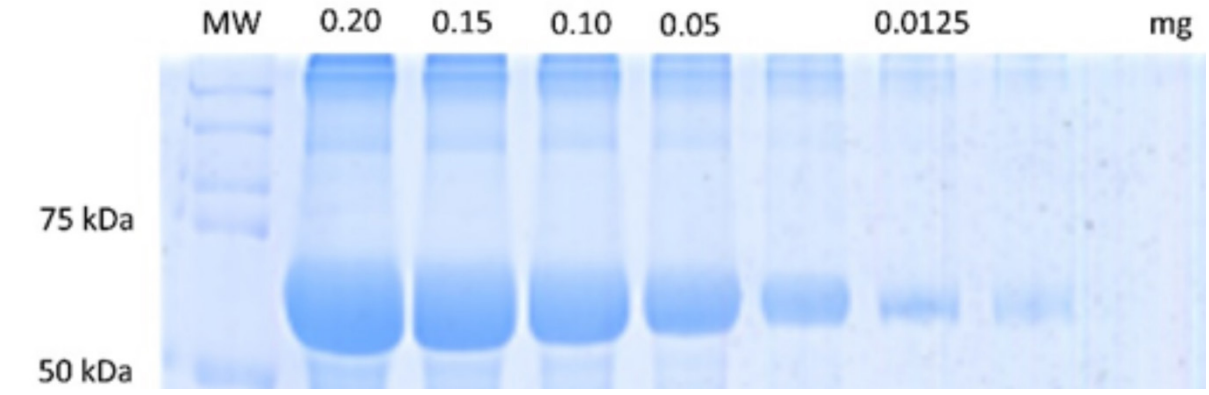

FIGURE 3 - Representation of the BSA calibration curve. Mean values of all 6 curves: $y = 0.28695x + 9.583867$, $R^2 = 0.9956$.

Figure 4 shows the electrophoresis gel analysis of papain proteolytic activity. According to the results, the enzyme activity was maintained in HGP10 but lost in HGWA.

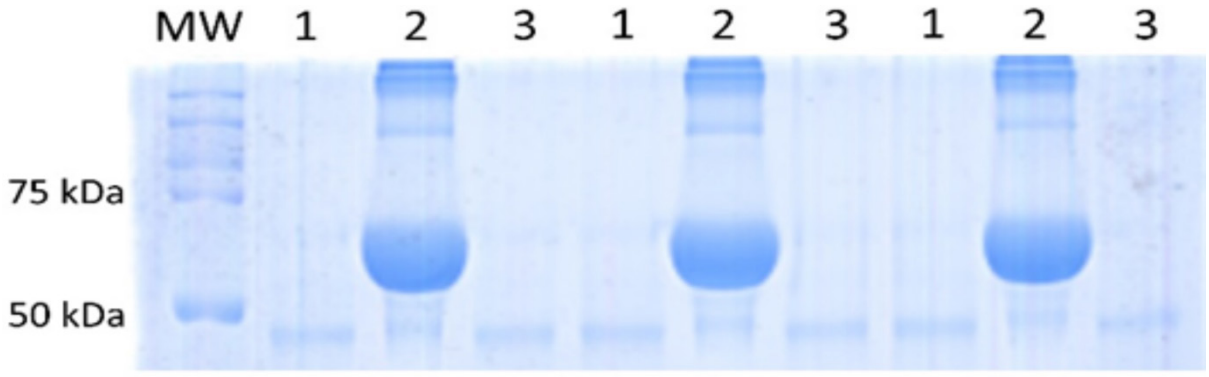

FIGURE 4 - Electrophoresis gel analysis of papain proteolytic activity: MW) Molecular weight; 1) HGP10; 2) HGWA; 3) HGSPU. Repeated numbers: triplicates. Analysis: T7, 2-8ºC. 10 µl of BSA (20 mg/mL) were added to each well.

The enzyme stability results are shown in Figure 5. Stability is measured by BSA consumption in study time and according to temperature.

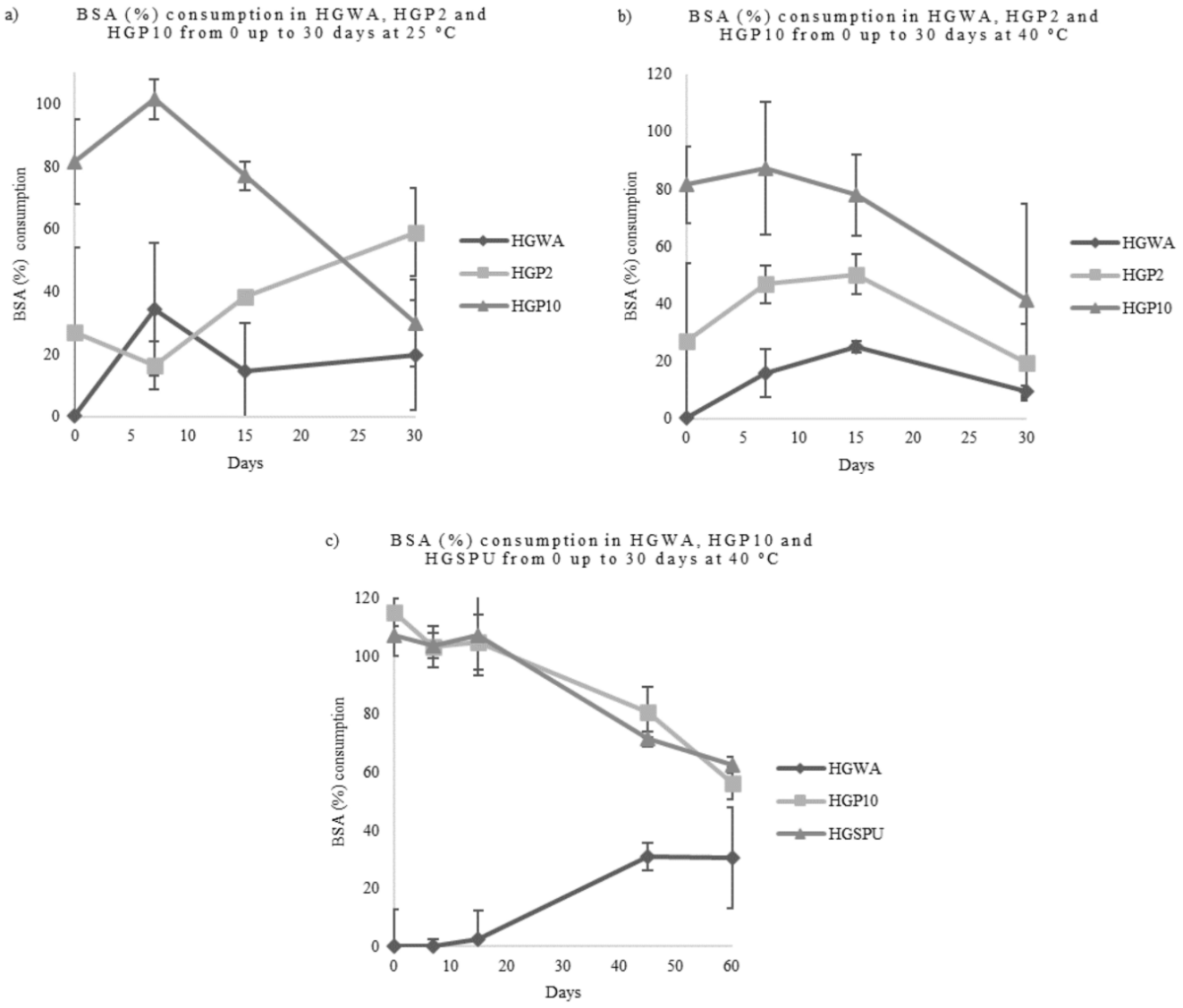

FIGURE 5 - Graphs of BSA consumption by study time and according to temperature: a) HGWA, HGP2, and HGP10 for 30 days at 25ºC; b) HGWA, HGP2 and HGP10 for 30 days at 40ºC; c) HGWA, HGP10 and HGSPU for 60 days at 2-8ºC.

Evaluation of clinical efficacy

Figure 6 shows the survival curves related to healing time. The Kaplan-Meier product estimator was used to prepare the curves.

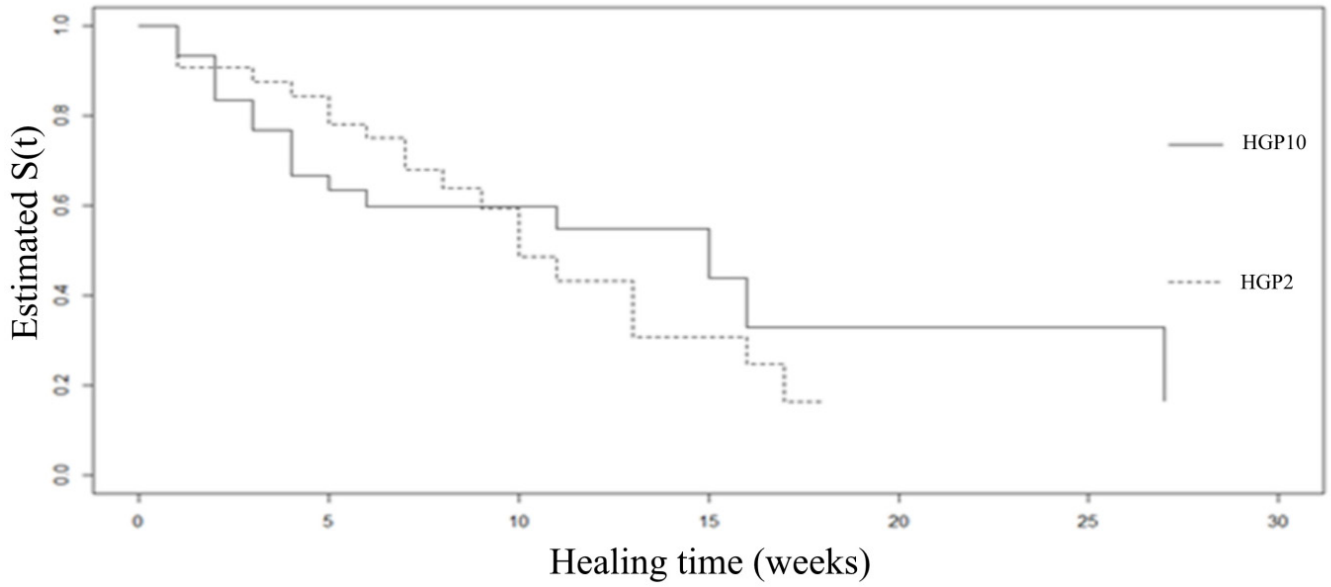

FIGURE 6 - Survival curves related to healing time (weeks) according to the formulations used: HGP2 and HGP10.

Table V shows the Log-rank test used to verify the statistical difference between the survival curves of both groups: HGP10 and HGP2. Thus, the hypothesis was null and there were no differences in the survival distributions between the groups.

TABLE V - Log-rank test used in the comparison between the survival curves obtained in the groups treated with the HGP2 and HGP10 formulations

Formulation	n[1]	Observed[2]	Expected	Log-rank test	p-value
HGP10	30	16	17.1	0.2	0.7
HGP2	32	19	17.9		

[1]Number of patients in the group; [2]Number of patients with wounds healed at the end of the study.

Figures 7 and 8 show the results of the Clinical Evolution of the patients treated (and healed) with the HGP2 and HGP10 formulations, respectively. Some problems related to suspected serious illness due to lack of adherence to the treatment and institutional logistical complications were identified.

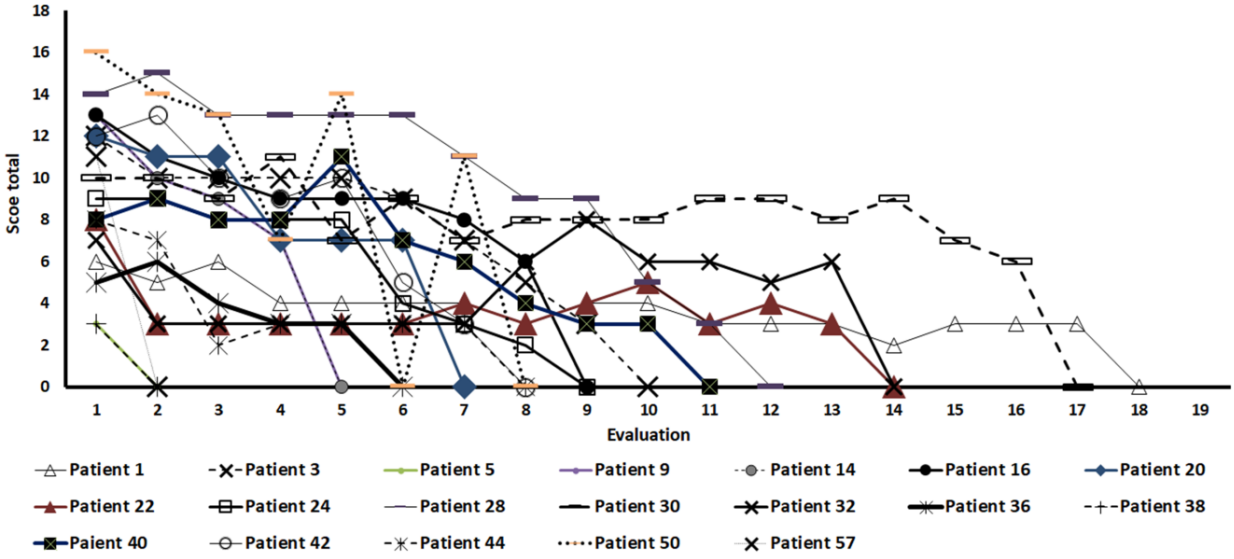

FIGURE 7 - Clinical evolution of the patients treated (and healed) with the HGP2 formulation throughout the clinical study, correlating the total score to the evaluation week.

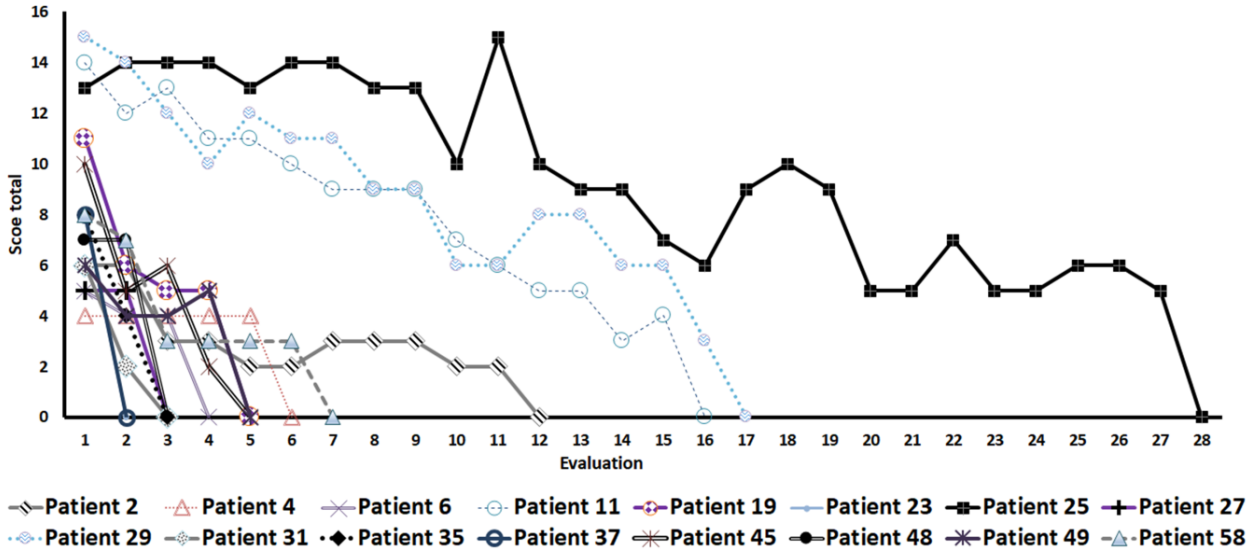

FIGURE 8 - Clinical evolution of the patients treated (and healed) with the HGP10 formulation throughout the clinical study, correlating the total score to the evaluation week.

DISCUSSION

Preparation of the hydrogels, characterizations and stability tests

The association of urea in formulations containing papain exposes the cysteine residues of its active site, facilitating the enzymatic action (Langer *et al.*, 2013). Urea also works for nonspecific debridement within a wide pH range (3.0-12.0), thus providing greater stability of the drug at room temperature. Urea should have at least half the concentration of papain and can vary from 0.5 to 2 in terms of the urea/papain ratio. To prevent urea from decomposing into ammonia, it is advisable to use the Boron compound as preservative. For sodium borate to stabilize urea at almost neutral pH,

it can be in the formulation of 0.5% to 10%, preferably 1%, as it is still soluble in water at this concentration (Panuncialman, Falanga, 2007). Another important advantage of employing urea is its moisturizing action when used in concentrations of up to 5% by weight of urea (Lodén, 2012; Fluhr, Cavallotti, Berardesca, 2008). As preservative for the entire formulation, phenoxyethanol was chosen for its use in several products, such as vaccines (Cosby, Stone, 2019), sexual lubricants (Shabir, 2010) and topical corticosteroids (Roy, Chakrabarty, 2013) that also fit into the category of topical medications, as well as the formulation presented in this article.

Determination of the pH of the hydrogels during the stability study (Table II) presented interesting results. The HGWA formulation showed a statistical difference between T0 and T7 at 25ºC, T0 and T15 at 25ºC and 40ºC, and T0 and T30 at 25ºC and 40ºC, as well as between T30 at 25ºC and T30 at 40ºC. In HGP2, there was no statistical difference between T0 and T7, unlike T0 and T15 for both temperatures. At 25ºC, T30 remained statistically similar to T0, differently from T30 at 40ºC. In HGP10 there was a statistical difference between T0 and T15 at 40ºC, as well as between T0 and T30 at 25ºC. However, there was no statistical difference between T30 at 40ºC and T0, or between T0 and T7 of both formulations. Both in HGP2 and HGP10 it is observed there was no difference between the T0 and T7 values at 25ºC and 40ºC, which demonstrates the 1-week stability of these formulations. The pH of the hydrogels remained around 5, slightly acidic, an ideal value for topical formulations (Boer *et al*., 2016; Schneider *et al*., 2007).

The density measurement (Table III) during the stability study presented interesting results. In HGWA, this difference was present ($p<0.0001$) comparing the results obtained in T0 with those obtained in T30 both at 25ºC and at 40ºC, which are similar to each other. Another statistical difference was found between T15 at 25º and T15 at 40ºC. In HGP2, statistical differences ($p<0.0001$) were found between T0 and T7 at 25ºC, between T0 and T15 at both temperatures, and between T0 and T30 at 40ºC. In HGP10, T0 and T7 are statistically different ($p = 0.0010$) at both temperatures, and T0, T15 and T30 at 40ºC. In both formulations containing papain and urea (HGP2 and HGP10) there was a statistical difference after one week (T7) of conditioning at 25°C (HGP2) and 40°C (HGP10). Therefore, the presence of papain and/or urea may be causing instability since HGWA did not present this characteristic in T7. It is also observed that the HGWA density showed values below 1 at T30, which was not the case in HGP2 and HGP10. "Formulations commercialized without urea addition are refrigerated and require temperature control during transportation and conservation in order to maintain their physical and chemical properties, a situation that was not necessary in this study, facilitating logistics and reducing refrigeration costs (Martins *et al*., 2011)". The formulations evaluated in this study are for dermatological use and treatment of areas that naturally need hydration (Lodén, 2012; Fluhr, Cavallotti, Berardesca, 2008). Therefore, formulations with density values below 1 can mean loss of moisture, which would not be less beneficial for wound treatment.

Rheological characterization

The flow and viscosity curves (Figure 1) were generated in the rotational tests. These measurements were monitored at 5ºC, 20ºC and 32°C. The initial apparent viscosity values for samples HGWA, HGP2 and HGP10, respectively, were: (i) 281.0 ± 2.8, 186.7 ± 7.0, and 172.0 ± 8.9 Pas at 5°C; (ii) 177.5 ± 7.8, 190.5 ± 6.4, and 167.7 ± 5.7 Pas at 20°C and (iii) 281.0 ± 5.2, 189.5 ± 12.0, and 165.3 ± 7.4 Pas at 32°C. These results indicated no statistically significant variation ($p = 0,0748$) in the sample viscosities values. This behavior suggests good physical stability of the formulations in the wide temperature range studied. Additionally, there was no change in the profiles of the rheograms for the samples at different temperatures. Figures 1-A*, 1-B* and 1-C* show that the flow type is non-Newtonian and pseudoplastic. Nonlinearity of the stress-strain rate ratio is characterized by the flow index, which measures the shear-thinning degree ($n<1$) in the Herschel-Bulkey model, commonly used for describing gels (Di Giuseppe, 2015; Kim *et al*., 2003).

The loss and storage moduli were determined to evaluate the viscoelastic behavior of the hydrogels. Considering that the viscosity values and profiles did not show significant differences between HGP2 and HGP10, the influence of the active component in the formulation

was only compared between HGWA and HGP10 at 20°C. In addition, concentration of the polymer in the hydrogel remains constant, which could significantly change strength of the gel. The measuring results of amplitude sweeps were presented with strain plotted on the x-axis and the storage G' and loss G'' moduli plotted on the y-axis, with both axes on a logarithmic scale (Figure 2). The G' values were greater than G'' for both samples, i.e., G'>G'', meaning that the elastic behavior dominates the viscous behavior, showing predominance of the elastic effects (solid-like gels). Table IV shows the tanδ (G''/G') values, which measures how elastic (tanδ < 1) the sample is, were 9.63 e^{-2} and 8.71 e^{-2} for HGWA and HGP10, respectively, and showed close values for tanδ (G''/G'). In the conditions studied, no crossover (G'= G'' or tanδ =1) was observed for the formulations. Therefore, it was not possible to determine the value of deformation and shear stress at the gel-sol transition point or the flow point.

The LVE region corresponds to the strain range, at a constant frequency, in which the sample structure remains unchanged and the G' and G'' curves are parallel. Therefore, the rheogram in Figure 2 indicates a decay that corresponds to the LVE region limit or linearity limit. At this point, the strain limit (γ_L) is calculated in percentage, as well as the yield point ($\square_y$) and the shear stress value, in Pa (Table IV). At this point, the material "softens" under shear. As shown by the data in Table IV, the LVE region limit is earlier for HGP10 than for HGWA, and HGP10 also presented a smaller γL percentage as a result of less effort to possibly make sol-gel structure disturbance, as it was not a transition. The presence of papain probably diminishes the transition strength. The samples retain the properties of the solid matter after this point, indicating good gel stability (Anton Paar, 2020).

Papain proteolytic activity evaluation

Proteolysis of BSA (66.5 kDa) was quantitatively analyzed by means of electrophoresis in a polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) through a densitometry scan. This technique was chosen due to its versatility, handling ease, good cost-benefit ratio and effectiveness for the analysis and separation of proteins (Sahoo *et al*., 2012; Lee, Kim, Lee, 2019), in addition to the possibility of simultaneously analyzing multiple samples under the same conditions (Maurye *et al*., 2018).

Validation of the methodology for evaluating papain proteolytic activity was performed using six BSA calibration curves in SDS-PAGE (Figure 3). The minimum amount of BSA to be detected by means of this method, limit of detection (LoD), was $6.08.10^{-4}$ mg of BSA; whereas the minimum amount of BSA that can be quantified with precision and accuracy, the limit of quantitation (LoQ), was $1.84.10^{-3}$ mg.

The papain present in the HGP10 formulation was able to selectively degrade BSA, observed in 66.5 kDa, while we did not observe proteolysis in the presence of HGWA (Figure 4). For intra-day precision, at BSA levels of 0.05 mg, 0.10 mg and 0.20 mg, it was 0.047 mg ± 0.00052, 1.11%; 0.103 mg ± 0.0028, 2.75%; and 0.197 mg ± 0.0014, 0.72%, respectively. Intermediate precision, at the BSA levels of 0.05 mg, 0.10 mg and 0.20 mg, was 0.049 mg ± 0.0036, 7.42%; 0.103 mg ± 0.0036, 3.46%; and 0.195 mg ± 0.0025, 1.28%, respectively. In both analyses, the Relative Standard Deviation (RSD) was considered satisfactory, as it presented values below 5.0, according the minimum acceptable criterion (ANVISA, 2017). It was observed that, at all three BSA levels (0.05 mg, 0.10 mg and 0.20 mg), the mean results of the recovery tests were 93.84% ± 1.11%, 103.18% ± 2.75% and 98.66% ± 0.72%, respectively. The accuracy based on the recovery method showed RSD values below 5%, corroborating that the method used in this study is effective in quantifying the activity of papain in the gels (ANVISA, 2017).

The enzyme stability results are shown in Figure 5. At time 0, the HGWA formulation did not show degradation of albumin: this means there was no proteolytic action. The HGP2 and HGP10 formulations in Figures 5a and 5b showed BSA degradation, even when compared, consistent with their papain concentrations (2% and 10%, respectively). After T7, both HGP2 and HGP10 showed a reduction in BSA consumption. An increase in BSA consumption in HGP2 and HGWA at 25ºC is observed in the final times, as was the case of HGWA at 2-8ºC (Figure 5c). This can demonstrate that, even without active formulation, it has some proteolytic action or that, possibly, some degradation of the material caused this fact. In Figure 5c, when comparing BSA consumption

between HGP10 and HGSPU, both follow a decay curve until the end of the study, which indicates that the formulation studied (HGP10) would have a profile close to the one already commonly used for the treatment of wounds (HGSPU), when stored under similar conditions.

Evaluation of clinical efficacy

The patients in the clinical study were admitted on different dates to the outpatient service of the Skin Integrity Methods Commission (*Comissão de Métodos relacionados à Integridade da Pele*, COMEIP) at HUCFF, and evaluated for different periods (weeks), according to the individual healing processes, the minimum number being maximum ratings of 2 and 28, respectively. All 62 patients with lower limb ulcers that participated in the study were divided into two groups: treated with the HGP2 formulation (n = 32) or treated with the HGP10 formulation (n = 30). However, not all patients reached the end of the clinical study.

Regarding the variables corresponding to the profile of the patients included in the study, it was found that: 61.3% were female; 53.2% were white-skinned, 29.0% were black-skinned and 17.7% were brown-skinned; 58.1% had injury times of less than six months; 66.1% of the patients had venous ulcers secondary to Chronic Venous Insufficiency (CVI); 4.9% of the patients had neuropathic ulcers secondary to diabetes *mellitus* and 29.0% had neuropathic ulcers secondary to Hansen's disease; and 66.1% were older adults. The Chi-square test was used to assess homogeneity between the variables of both groups, and no statistical difference was observed at the 5% significance level. Consequently, there was no difference between the profiles of the patients in both groups.

According to Kleinbaum and Klein (2012), clinical trials usually use survival analysis models in a specific period, for a given event to occur, it is considered the variable of interest (Kleinbaum, Klein, 2012). This model is adopted, for example, in assessments of the time until onset of a particular disease, its cure or recurrence. However, information about the timing of events that have not completely ended, called incomplete or censored times, should also be considered in this analysis.

For the survival analysis, time was observed in weeks, from the patients' inclusion in the clinical study to healing of their wounds in both groups, and this was initially considered as the event of interest, also including censored data. The Kaplan-Meier product estimator was used to prepare the survival curves for the healing time of all patients (Figure 6).

It was observed that the mean time for wound healing with the HGP2 and HGP10 formulations was 10.7 and 12.8 weeks, respectively. The Log-rank test was used to verify the statistical difference between the survival curves of both groups, as shown in Table V, considering the null hypothesis that there were no differences in the survival distributions between the groups.

The statistical analysis showed that, considering p-value = 0.7, there was not enough evidence to reject the null hypothesis. The PUSH instrument, version 3.0, was used for the weekly assessment of healing evolution in the patients who used the HGP2 and HGP10 formulations. During their follow-up, 26 needed to interrupt their treatment due to 3 reasons: 3 of them for suspected serious illness (osteomyelitis, squamous cell carcinoma and peripheral obstructive arterial disease), requiring medical referral; 14 due to non-adherence to the treatment, and 09 due to institutional logistical complications (Figures 7 and 8).

Both formulations are effective in healing processes. However, only volunteers that used the HGP10 formulation did not experience adverse reactions or complications to the newly formed tissue in the wound bed. Regarding the control of clinical signs of wound infection, both formulations were effective, although *in vitro* studies only describe 10% papain as a bactericidal and bacteriostatic agent (Sibbald, Woo, Ayello, 2006). There is no consensus in application of a clean or sterile technique for wound care (acute and chronic) (Kent *et al*., 2018; Wound, Ostomy and Continence Nurses Society [WOCN] Wound Committee and Association for Professionals in Infection Control and Epidemiology, Inc. [APIC] 2000 Guidelines Committee, 2012) and, in this study, the clean technique was performed, achieving positive results.

The volunteers reported easy application of the formulations, as well as good spreadability and absorption by the wound. No undesirable effects or any other

type of clinical complications were observed during the applications, such as damage to the newly formed tissue, pain, bleeding or regression in evolution of the wound. Thus, the hydrogel was able to grant autonomy to the patients, enabling self-care, as they were able to change the daily dressings without the help of a health professional. This formulation also democratizes the use of phytomedication nationwide in hospitals, outpatient services, clinics, long-term institutions, homes and communities without electrical infrastructure, even for 7 days. It is also worth mentioning that the follow-up of outpatients at the hospital where the clinical trial was carried out also lasted 7 days.

The formulations developed showed an indication of storage at room temperature. The patients, especially those with venous insufficiency, reported not feeling pain during the application of the HGP2 and HGP10 formulations, a fact that is commonly observed when the medication used is kept refrigerated.

CONCLUSION

According to the test results, formulations HGWA, HGP2 and HGP10 are stable for 7 days at 25ºC. The study of proteolytic activity with the electrophoresis method proved to be promising and with satisfactory validation results. For the patients' well-being, the rheological characteristics of the non-Newtonian formulation and no yield-point were satisfactory for use in gel form. HGP2 and HGP10 did not present significant differences regarding the mean time for lesion healing. Both were effective and all patients who did not abandon treatment achieved a cure. However, HGP10 proved to be more efficient, as it does not cause setbacks in evolution of the healing process and can be used in all types of tissue present in the wound bed.

FUNDING

This paper was funded by *Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro* (FAPERJ) Carlos Chagas Filho – Support for *Stricto Sensu* Graduate Programs and Courses in the State of Rio de Janeiro [Grant number: E-26/210.136/2021] and by *Cientista do Nosso Estado* (CNE) [Grant number: E-26/201.077/2021], Rio de Janeiro, Brazil

ACKNOWLEDGEMENTS

The authors would like to thank the University pharmacy at the Federal University of Rio de Janeiro for their structural support in development of this project.

ABBREVIATIONS

BSA - Bovine Serum Albumin
G′ - Storage modulus
G″ - Loss modulus
Ho - Null hypothesis
H1 - Alternative hypothesis
HGP2 - Hydrogels containing 5% Urea and 2% Papain
HGP10 - Hydrogels containing 5% Urea and 10% Papain
HGWA - Hydrogels without Papain or Urea
HGSPU - Hydrogels containing 10% of papain but without urea or sodium borate
KEGG - Kyoto Encyclopedia of Genes and Genomes
LoD – Limit of Detection
LoQ - Limit of Quantification
PUSH - Pressure Ulcer Scale for Healing
SDS - Dodecyl Sulfate Sodium
SDS-PAGE - Gel of Dodecyl Sodium Sulfate Polyacrylamide

REFERENCES

Abbade LPF, Lastória S, Almeida RH. Venous ulcer: clinical characteristics and risk factors. Int J Dermatol. 2011;50(4):405–411.

Abdelrahman T, Newton H. Wound dressings: principles and practice. Surgery (Oxford). 2011;29(10):491–495.

Ahmed EM. Hydrogel: Preparation, characterization, and applications: A review. J Adv Res. 2015;6(2):105–121.

Akash MSH, Rehman K. Recent progress in biomedical applications of Pluronic (PF127): Pharmaceutical perspectives. J Control Rel. 2015;209:120–138.

Anton Paar GmbH. Amplitude sweeps. [citad 2020 October 19]. Available from: https://wiki.anton-paar.com/br-pt/varreduras-de-amplitude/.

ANVISA (National Health Surveillance Agency). Resolution N.166 (2017). criteria for the validation of analytical methods. [citad 2020 October 06]. Available from: https://www.in.gov.br/materia/-/asset_publisher/Kujrw0TZC2Mb/content/id/19194581/do1-2017-07-25-resolucao-rdc-n-166-de-24-de-julho-de-2017-19194412.

ANVISA (National Health Surveillance Agency). Resolution N.67 (2007). Disposes for good practices in manipulation of magistral and workshop preparations for human use in pharmacies. [citad 2021 May 14] Available from: https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2007/rdc0067_08_10_2007.html

Boer M, Duchnik E, Maleszka R, Marchlewicz M. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. Postepy Dermatol Alergol. 2016;33(1):1–5.

Brazilian Pharmacopeia. National Health Surveillance Agency (ANVISA). Six edition. Brasilia, Federal District: Anvisa; 2019. [citad 2020 October 28]. Available from: http://portal.anvisa.gov.br/documents/33832/259143/Volume+I+Pronto.pdf/4ff0dfe8-8a1d-46b9-84f7-7fa9673e1ee1.

Brunelle JL, Green R. One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE). In: Jon Lorsch, edithor. Methods in Enzymology (541), 1rs Edition. San Diego: Academic Press. 2014:151–159.

Buchmann S. Main cosmetics vehicles. In: Barel AO, Paye M, Maibach HI, edithors. Handbook of cosmetic science and technology. New York: Marcel Dekker Inc., 2001:145-170.

Cosby A, Stone, J. Immune-mediated adverse reactions to vaccines. Br J Clin Pharmacol. 2019;85(12):2694-2706.

Das A, Kumar A, Patil NB, Viswanathan C, Ghosh D. Preparation and characterization of silver nanoparticle loaded amorphous hydrogel of carboxymethylcellulose for infected wounds. Carbohydr Polm. 2015;130:254–261.

Das S, Baker AB. Biomaterials and nanotherapeutics for enhancing skin wound healing. Front Bioeng Biotechnol. 2016;4(82):1-20.

Di Giuseppe E. Characterization of Carbopol® hydrogel rheology for experimental tectonics and geodynamics. Tectonophysics. 2015;642:29–45.

Das A, Kumar A, Patil NB, Viswanathan C, Ghosh D. Preparation and characterization of silver nanoparticle loaded amorphous hydrogel of carboxymethylcellulose for infected wounds. Carbohydr Polm. 2015;130:254–261.

Fernández-Lucas J, Castañeda D, Hormigo, D. New trends for a classical enzyme: Papain, a biotechnological success story in the food industry. Trends Food Sci Technol. 2017;68:91-101.

Fluhr JW, Cavallotti C, Berardesca E. Emollients, moisturizers, and keratolytic agents in psoriasis. Clin Dermatol. 2008;26(4):380–386.

Formulário Nacional of the Brazilian pharmacopeia. National Health Surveillance Agency (ANVISA). Second edition Brasilia, Federal District: Anvisa; 2012. Available from: https://www.gov.br/anvisa/pt-br/assuntos/farmacopeia/formulario-acional/arquivos/8065json-file-1

Gainza G, Villullas S, Pedraz JL, Hernandez RM, Igartua M. Advances in drug delivery systems (DDSs) to release growth factors for wound healing and skin regeneration. Nanomed Nanotechnol. 2015;11(6):1551–1573.

Goldman B, Yorktown Heights NY, Rystan Company, Mount Vernon NY, 1959, Stable aqueous papain topical compositions, US2917433A.

Grip J, Engstad RE, Skjæveland I, Škalko-Basnet N, Holsæter AM. Sprayable Carbopol hydrogel with soluble beta-1,3/1,6-glucan as an active ingredient for wound healing: Development and in-vivo evaluation. Eur J Pharm Sci. 2017;107:24–31.

KEGG: Kyoto Encyclopedia of Genes and Genomes. Papain. [citad 2020 october 20]. Available from: https://www.genome.jp/kegg/.

Kent DJ, Scardillo JN, Dale B, Pike C. Does the use of clean or sterile dressing technique affect the incidence of wound infection? J Wound Ostomy Continence Nurs. 2018;45(3):265–269.

Kim J-Y, Song J-Y, Lee E-J, Park S-K. Rheological properties and microstructures of Carbopol gel network system. Colloid Polym Sci. 2003;281:614–623.

Kleinbaum DG, Klein M. Survival analysis a self-learning text. 3rd ed. New York: Springer-Verlag; 2012:1-700.

Larouche J, Sheoran S, Maruyama K, Martino MM. Immune regulation of skin wound healing: mechanisms and novel therapeutic targets. Adv Wound Care. 2018;7(7):209–231.

Lantis J, Paredes J. Permissive maintenance debridement -- the role of enzymatic debridement in chronic wound care. Wounds Int J. 2017;8(2):7–13.

Langer V, Bhandari PS, Rajagopalan S, Mukherjee MK. Enzymatic debridement of large burn wounds with papain–urea: Is it safe? Med J Armed Forces India. 2013;69(2):144–150.

Lee M-K, Kim JK, Lee S-Y. Effects of fermentation on SDS-PAGE patterns, total peptide, isoflavone contents and antioxidant activity of freeze-thawed tofu fermented with Bacillus subtilis. Food Chem. 2019;249:60–65.

Lodén M. Effect of moisturizers on epidermal barrier function. Clin Dermatol. 2012:30(3):286–296.

Mandelbaum SH, Di Santis EP, Mandelbaum MHS. Healing: current concepts and auxiliary resources - Part I. An Bras Dermatol. 2003;78(4):393-408.

Martins MD, Fernandes KPS, Pavesi VC, França CM, Mesquita-Ferrari RA, Bussadori SK. Healing properties of papain-based gel on oral ulcers. Braz J Oral Sci. 2011;10(1):120-123.

Maurye P, Dhabi M, Biswas JK, Bandyopadhyay TK. An integrated system for simultaneous casting of multi-polyacrylamide gels with varied concentrations. Measurement. 2018;114:274–285.

National Health Surveillance Agency. Resolution N.1. (2005) Guide for conducting stability studies. [citad 2020 October 06]. Available from: http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2005/res0001_29_07_2005.html.

National Pressure Ulcer Advisory Panel, European Pressure Ulcer Advisory Panel and Pan Pacific Pressure Injury Alliance. Prevention and Treatment of Pressure Ulcers: Quick Reference Guide. Emily Haesler (Ed.). Cambridge Media: Osborne Park, Australia; 2014.

Okur ME, Karantas ID, Şenyiğit Z, Okur NÜ, Siafaka PI. Recent trends on wound management: New therapeutic choices based on polymeric carriers. Asian J Pharm Sci. 2020;15(6):661-684.

Oliveira CA, Gouvêa MM, Antunes GR, de Freitas ZZF, Marques FFC, Ricci-Junior E. Nanoemulsion containing 8-methoxypsoralen for topical treatment of dermatoses: Development, characterization and ex vivo permeation in porcine skin. Int J Pharm. 2018;547(1-2):1–9.

Oliveira BGRB, Castro JBA, Granjeiro JM. Epidemiologic and clinical overview of patients with chronic wounds treated at ambulatory. UERJ Nursig J. 2013;21(5):612–617.

Panuncialman J, Falanga, V. The science of wound bed preparation. Clin Plast Surg. 2007;34(4):621–632.

Powers JG, Higham C, Broussard K, Phillips TJ. Wound healing and treating wounds: Chronic wound care and management. J Am Acad Dermatol. 2016;74(4):607–625.

Reichel C, Thevis M. Gel electrophoretic methods for the analysis of biosimilar pharmaceuticals using the example of recombinant erythropoietin. Bioanalysis. 2013;(5):587–602.

Roy C, Chakrabarty J. Development and validation of a stability-indicating RP-HPLC method for the simultaneous determination of phenoxyethanol, methylparaben, propylparaben, mometasone furoate, and tazarotene in topical pharmaceutical dosage formulation. Sci Pharm. 2013;81(4):951-67.

Sankalia MG, Mashru RC, Sankalia JM, Sutariya VB. Papain entrapment in alginate beads for stability improvement and site-specific delivery: Physicochemical characterization and factorial optimization using neural network modeling. AAPS Pharm Sci Tech. 2005;6(2):E209–E222.

Santos VLCG, Azevedo MAJ, Silva TS, Carvalho VMJ, Carvalho VF. Crosscultural adaptation of the pressure ulcer scale for healing to the portuguese language. Lat Am J Nurs. 2005;13(3):305–313.

Shabir GA. development and validation of a rplc method for the determination of 2-phenoxyethanol in senselle lubricant formulation. Indian J Pharm Sci. 2010;72(3):307-311.

Sibbald RG, Woo K, Ayello EA. Increased bacterial burden and infection: the story of NERDS and STONES. Adv Skin Wound Care. 2006;19(8):447–461.

Thierry A, Maillard MB, Lortal S. Detection of aminotransferase activity of Propionibacterium freudenreichii after SDS-PAGE. J Microbiol Methods. 2002;51(1);57–62.

Tricco AC, Antony J, Vafaei A, Khan PA, Harrington A, Cogo E, et al. Seeking effective interventions to treat complex wounds: an overview of systematic reviews. BMC Med. 2015;13:89.

Varaprasad K, Raghavendra GM, Jayaramudu T, Yallapu MM, Sadiku RA. Mini review on hydrogels classification and recent developments in miscellaneous applications. Mat Sci Eng C. 2017;79:958–971.

Wound, Ostomy and Continence Nurses Society (WOCN) Wound Committee, Association for Professionals in Infection Control and Epidemiology, Inc. (APIC) 2000 Guidelines Committee. Clean vs. sterile dressing techniques for management of chronic wounds: a fact sheet. J Wound Ostomy Continence Nurs. 2012:39(2 suppl):S30-34.

Yamamoto E, Yamaguchi S, Nagamune T. Effect of β-cyclodextrin on the renaturation of enzymes after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Anal Biochem. 2008;381(2):273–275.

Zhao R, Liang H, Clarke E, Jackson C, Xue M. Inflammation in chronic wounds. Int J Mol Sci. 2016;17(12):2085.

Zhao GH, Kapur N, Carlin B, Selinger E, Guthrie JT. Characterisation of the interactive properties of microcrystalline cellulose–carboxymethyl cellulose hydrogels. Int J Pharm. 2011;415(1-2):95–101.

Received for publication on 19th December 2020
Accepted for publication on 15th March 2022