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Article

Association of *Rhizophora mangle* and ascorbic acid in hydrogels: Evaluation of cytotoxic and immunomodulatory effects

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Polyphenolics from *Rhizophora mangle* (*R. mangle*) have shown wound healing and anti-inflammatory effects that may be potentiated by being associated with ascorbic acid, an important substance for collagen and elastin synthesis that plays a role in tissue repair. In our study, we aimed to formulate an association of *R. mangle* and ascorbic acid in hydrogels and evaluate the association's cytotoxic and immunomodulatory effects. In a pre-formulation study, three gelling polymers (i.e.xanthan gum, poloxamer and hydroxyethyl cellulose) were tested. The selected polymer (i.e. xanthan gum) was used to evaluate cytotoxic and immunomodulatory effects using flow cytometry. Xanthan gum (1.5%) had a homogeneous appearance, an orange colour, a smooth surface, intense brightness and the typical odour, as well as non-Newtonian pseudoplastic behaviour. With a pH of 5.0–5.3 and a non-cytotoxic profile, xanthan gum induced the proliferation and activation of $CD4^+$, $CD8^+$ and NK T lymphocytes and the production of IL-2, IL-4, IL-10, IL-17 and TNF-α cytokines in stimulated splenocytes. The results suggest that the association of *R. mangle* and ascorbic acid in 1.5% xanthan gum hydrogel may be promising in preparations for wound-healing processes.

Keywords: *Rhizophora mangle*. Ascorbic acid. Hydrogel. Healing. Cytotoxicity. Immunological factors.

INTRODUCTION

Rhizophora mangle L., popularly known as red mangrove, is an endemic species of mangrove vegetation belonging to the *Rhizophoraceae* family (Regalado, Sánchez, Mancebo, 2016). The plant's pharmacological properties stem from the presence of secondary compounds that it contains—namely, the polyphenolics described in the works of Regalado, Sánchez, Mancebo (2016) and Oliveira (2018) as p-coumaric phenolic acid, quinic acid, gallic acids, ellagic and chlorogenic acids, gentisaldehyde, catechin and epicatechin—all of which contribute to biological activities that facilitate improved wound healing, have antioxidant and anti-inflammatory effects and promote neo-angiogenesis (Marrero *et al*., 2006; Ofori-kwakye, Kwapong, Bayor, 2001).

Studies have shown the incorporation of *R. mangle* leaf extract into formulations and biomaterials such as gels, creams and skin substitute films used in tissue repair in the case of wounds, burns and ischemic skin flaps (Roger *et al*., 2011; Araújo, 2015; Oliveira, 2018). Several dressing materials on the world market can

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be used in the different stages of wound treatment, including alginate, activated charcoal, silver plates, collagen matrices, cellulose matrices, transparent films and hydrogels (Smaniotto *et al.*, 2012). Among them, hydrogels are formed by polymers such as xanthan gum, hydroxyethyl cellulose and poloxamer 407 and used to develop dressings aimed at treating wounds given favourable properties such as permeability to water and metabolic materials, flexibility and durability, as well as the ease and low cost of their development (Dumortier *et al.*, 2006; Mohsin, Shaikh, 2017).

However, the application of hydrogels is not restricted to the development of dressings. Studies have also described the use of hydrogels as biomaterials in cell cultures and in endovascular applications, cell transplantation, enzyme immobilisation and even the development of a controlled drug delivery system (Rubira *et al.*, 2009; Sixiang *et al.*, 2018; Xiaosai *et al.*, 2018; Mazhar *et al.*, 2019).

Combining compounds to accelerate the healing effect is an important practice in the development of formulations (Pessoa, 2014). Biaou *et al.* (2020) have described a dressing that acts to control fibrosis as well as improve the healing process in wounds and drawn particular attention to its composition, formed by combining silk sericin and surface micropatterns in cellulose bacteria. In this article, the association of ascorbic acid in formulations is proposed to improve the synthesis of collagen and elastin and thus promote favourable conditions for tissue repair, namely by configuring its topical use as an important therapeutic strategy to accelerate the healing of wounds (Pessoa, 2014). Chronic wounds in Brazil continue to cause serious problems for public health due to the large number of people with impaired skin integrity and the difficulty of treatment, which burdens public spending and causes personal, social, psychological and economic damage (Silva *et al.*, 2017). Although the process of wound healing has begun to be understood more widely, there remains a need for further studies that evaluate its mechanisms and provide directions for more efficient preventive and healing measures (Campos, Borges-Branco, Groth, 2007). After all, information about those aspects can encourage the development of new formulations that combine substances in order to accelerate the healing process. Considering all of the above, in our study we aimed to develop hydrogels based on *R. mangle* 5% and ascorbic acid 5% in a 10% association to evaluate their cytotoxic and immunomodulatory effects.

MATERIAL AND METHODS

Material

Freeze-dried aqueous extract of *R. mangle* was obtained from the Department of Histology and Embryology at Universidade Federal de Pernambuco (UFPE). The phytochemical characterisation of *R. mangle* has previously been described by Oliveira (2018). Meanwhile, the materials for hydrogel formulations (i.e. polymers, ascorbic acid, sodium metabisulfite, methylparaben and propylparaben) were supplied by Sigma Chemical Company (USA). In a pre-formulation study, three gelling agents were used in different concentrations: hydroxyethyl cellulose, xanthan gum and poloxamer 407 (Pluracare®) (Table I).

TABLE I - Composition of formulations containing extract of *R. mangle* leaves and ascorbic acid

Composition (%; P/V)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
R. mangle* extract *(%)	5	5	5	5	5	5	5	5	5	5
Metabisulfite sodium (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Methylparaben (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Propylparaben (%)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

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Composition (%; P/V)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Natrosol (%)	1	-	-	-	-	-	-	-	-	-
Xanthan gum (%)	-	1	1.5	2	-	-	-	-	-	-
Poloxamer 407 (%)	-	-	-	-	15	18	20	22.5	25	30
Ascorbic acid (%)	-	-	10	-	-	-	-	10	-	-
Distilled water q.s.p (g)	100	100	100	100	100	100	100	100	100	100

Note: F: formulation; %: concentration of compound in total formulation; P/V: compound weight by volume.

Study Design and Sample Collection

Our study involved a preclinical *in vitro* evaluation of the cytotoxic and immunomodulatory activities of hydrogels based on the association of *R. mangle* leaf extract and ascorbic acid in the splenocytes of mice. Five female BALB/c mice 6–8 weeks old were raised and maintained at the animal facilities of Keizo Asami Immunopathology Laboratory located at UFPE. Mice were kept under standard laboratory conditions (i.e.22 °C and 12 h day–night cycle) with free access to a standard diet (Labina/Purina, Campinas, Brazil) and water. All experimental procedures were performed in accordance with the Ethics Committee of Animal Use at UFPE (Protocol No.: 0048/2016). Splenocyte collection was performed following Aguiar *et al.*'s (2019) procedure. After euthanasia, the spleen of each mouse was removed aseptically and placed in a Falcon tube containing RPMI 1640 with foetal calf serum (i.e. complete medium). In a vertical flow, each spleen was transferred to a Petri dish and soaked, and the cell suspensions obtained from each spleen were transferred to Falcon tubes containing approximately 10 mL of incomplete medium. Spleen homogenates were overlaid onto a Ficoll-PaqueTM PLUS layer, with the density adjusted to 1.076 g/mL, and centrifuged at 1000× *g* at room temperature for 25 min. The interface cell layer containing immune cells was recovered using a Pasteur pipette, washed twice in phosphate-buffered saline (PBS) and centrifuged twice at 500× *g* for 10 min. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were used only when viability exceeded 95%.

Development of Hydrogels

Formulations containing xanthan gum and hydroxyethyl cellulose were obtained following the protocol of Mohsin, Shaikh (2017). The polymer (i.e. xanthan gum or hydroxyethyl cellulose) was dispersed in a beaker with distilled water for 30 min. The metabisulfite antioxidant was dissolved in distilled water at room temperature, while the methylparaben and propylparaben preservatives were dissolved in previously heated distilled water. After the polymer swelled, it was stirred for another 30 min at 750 RPM and gradually added to the solution containing antioxidant and homogenised preservatives. The aqueous extract of *R. mangle* was incorporated by dissolving it in distilled water and gradually adding it to the base of the stirring formulations at 750 RPM for homogenisation. Next, ascorbic acid was incorporated, and the mixture was stored at room temperature. Meanwhile, the formulations containing poloxamer 407 were obtained according to Dumortier *et al.* (2006). The antioxidant metabisulfite was dissolved in room-temperature distilled water, while the methylparaben and propylparaben preservatives were dissolved in previously warmed distilled water, after which the solution was homogenised. Poloxamer 407 was poured into the solution and left overnight in the refrigerator. Last, the association of the aqueous extract of *R.*

mangle and ascorbic acid (i.e. active association) was incorporated into the solution.

Pharmaceutical Evaluations

Major organoleptic characteristics were evaluated according to three criteria: appearance, colour and odour (Brasil, 2004). The pH of the formulations was determined using the pH 1800 model GE 1800 (GEHAKA®), previously calibrated with the pH 7.0 and pH 4.0 buffer solutions at 4 °C, without diluting the product (Prista *et al.*, 2008). Rheological behaviour was evaluated using a Rheology International rotational viscometer and data analysis using Microsoft® Excel software. With the construction of a viscosity versus shear rate graph, the rheological behaviour of the products was identified (Goebel, 2012). Spreadability was determined in triplicate by the adapted method of Borghetti, Knorst (2006). The results were recorded as sample spreadability as a function of the applied weight according to the equation:

$$Ei = d^2\pi / 4$$

Ei = sample spreadability for a weight *i* (mm^2); d = mean diameter (mm).

Cytotoxicity Assays

Analysis of cell viability using annexin V-FITC and propidium iodide staining

Mice splenocytes (i.e. 10^6 cells) were treated with the formulations in concentrations of 0.05%, 0.1% and 0.2% in 24-well plates for 24 h to analyse the cytotoxicity of the hydrogels. The procedure was performed following Aguiar *et al.*'s (2019) method. Untreated cells, only in RPMI 1640 medium, were used as a negative control. After lymphocytes were centrifuged at 26 °C and 450× *g* for 10 min, PBS 1× (1 mL) was added to the precipitate, which was centrifuged at 26 °C and 450× *g* for another 10 min. The pellet was resuspended in the binding buffer of a cell viability kit (Becton Dickinson Biosciences), and annexin V conjugated with fluorescein isothiocyanate (FITC) (1:500) and propidium iodide (PI, 20 µg/mL) was added to each labelled cytometer tube. Flow cytometry was performed in a FACS Calibur flow cytometer (Becton Dickinson Biosciences) and analysed using CellQuest Pro software (Becton Dickinson Biosciences). The results were analysed by using graphs (i.e. dot plots). Annexin–FITC-negative and PI-positive cells were considered to be necrotic, while Annexin–FITC-positive and PI-negative cells were considered to represent splenocytes in the early stage of apoptosis. Double negatives were considered to indicate viable cells.

Cell proliferation analysis using CFSE staining

Formulations in a concentration of 0.1% were analysed following Aguiar *et al.*'s (2019) method. The cell solution was centrifuged at 300× *g* at room temperature for 5 min with sterile PBS 1× supplemented with SFB 5% (pH 7.2). Afterwards, the cell solution was adjusted to 10 × 106 cells/mL and received 5 mM of 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE). Cells were incubated for 10 min at room temperature in a dark cycle and centrifuged twice at 300× *g* for 5 min with sterile PBS 1×. The stained cells were cultured for 24 and 48 h with the formulations in a concentration of 0.1% (i.e. treated group) or with RPMI 1640 medium (i.e. negative control). After culture, cells were centrifuged at 300× *g* for 5 min, and acquired on FACSCalibur platform (Becton Dickinson Biosciences) and the results were analysed using CellQuest Pro software (Becton Dickinson Biosciences).

Immunological assays

Cytokine production in mice supernatants

Supernatants of the splenocyte cultures treated or not treated with the formulations under study were evaluated according to Filho *et al.*'s (2019) method. The supernatants were collected for the quantification of cytokines using the Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Biosciences) for the simultaneous detection of interleukins (i.e. IL-2, IL-4, IL-6, IL-10 and IL-17A), tumour necrosis factor (TNF) and interferon-

gamma (IFN-γ). The assays were performed according to the manufacturer's instructions, and data were acquired on the FACSCalibur platform. Seven individual cytokine standard curves (i.e. 0–5000 pg/mL) were run in each assay. The range of detection was between 2 and 5000 pg/mL. The results were analysed using FCAP 3.1 software (Becton Dickinson Biosciences).

Lymphocyte immunophenotyping assay

Lymphocytes and monocytes present among the splenocytes were cultured in RPMI medium for 24 h in 24-well plates at a density of 10^6 cells per well in the presence and absence of the formulations. They were evaluated according to the method adapted by Santos *et al.* (2018). After incubation, cells were removed from the plates using ice-cold PBS wash 1% and transferred to 15 mL polypropylene tubes (BD Biosciences) with 6 mL of PBS wash for centrifugation at 400× *g* for 10 min. After the supernatant was discarded, cells were washed with 2 mL of PBS wash and centrifuged at 400× *g* for 5 min. The supernatant was again discarded, and surface monoclonal antibodies were added to the tubes, which were subsequently incubated for 30 min. Two washing steps were performed with 1 mL of PBS wash followed by centrifugation at 400× *g* for 5 min. The supernatants were discarded once again, and cells were fixed for 15 min with 150 mL of Cytofix solution (BD Biosciences) and washed with 2 mL of PBS wash, followed by centrifugation at 400× *g* for 5 min. After the supernatant was discarded a final time, 300 µL of PBS wash was added to each tube, which was loaded onto the FACSCalibur platform. Monoclonal antibodies used were FITC Rat Anti-mouse CD4, PE Rat Anti-mouse CD8 and FITC Rat Anti-mouse CD16/CD32 (BD Biosciences).

Statistical Analysis

Data were analysed using non-parametric tests. To detect any differences between the groups, the Wilcoxon test was used, while Student's *t* test was used to analyse the results from the cell viability assay. All results were expressed as $M \pm SD$, and any value with $p < .0001$ was considered to be statistically significant.

RESULTS

Development of Hydrogels

Xanthan gum showed better compatibility with the active association (i.e. *R. mangle* and ascorbic acid) than hydroxyethyl cellulose and poloxamer 407. The concentration of xanthan gum chosen for pharmaceutical, cytotoxic and immunomodulatory evaluations was 1.5% represented by F3 (a, b, c, d, e, f, g) due to its better consistency and appearance (Table II).

TABLE II - Composition of xanthan gum 1.5% formulations containing extract of *R. mangle* leaves and ascorbic acid in different concentrations

Composition (%; P/V)	F3(a)	F3(b)	F3(c)	F3(d)	F3(e)	F3(f)	F3(g)
R. mangle* extract *(%)	-	-	5	5	5	5	5
Metabisulfite sodium (%)	0.2	1	0.2	0.2	0.2	1	1
Methylparaben (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Propylparaben (%)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Xanthan gum (%)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Ascorbic acid (%)	-	-	-	5	10	10	5
Distilled water q.s.p (g)	100	100	100	100	100	100	100

Note: F: formulation; %: concentration of compound in total formulation; P/V: compound weight by volume.

Pharmaceutical Evaluation

The formulations with xanthan gum had a homogeneous appearance, an orange colour, a smooth surface, intense brightness and an odour typical of the active association. The pH of the formulations studied was from 5.0 to 5.3, as shown in Table III. The formulations under study presented non-Newtonian pseudoplastic behaviour (Figure 1) that showed no statistically significant difference compared with the control. The spreadability of the formulations showed a gradual increase as a function of the weight (g) applied to the samples (Figure 2) but showed no statistically significant difference compared with the control.

TABLE III - Average pH values of formulations based on *R. mangle* and ascorbic acid

Parameter $n = 3$	F3(a)	F3(b)	F3(c)	F3(d)	F3(e)	F3(f)	F3(g)
pH ± *SD*	5.0 ± 0.1	5.1 ± 0.15	5.1 ± 0.15	5.2 ± 0.1	5.0 ± 0.05	5.1 ± 0.05	5.3 ± 0.07

Subtitle*:* F: formulation.

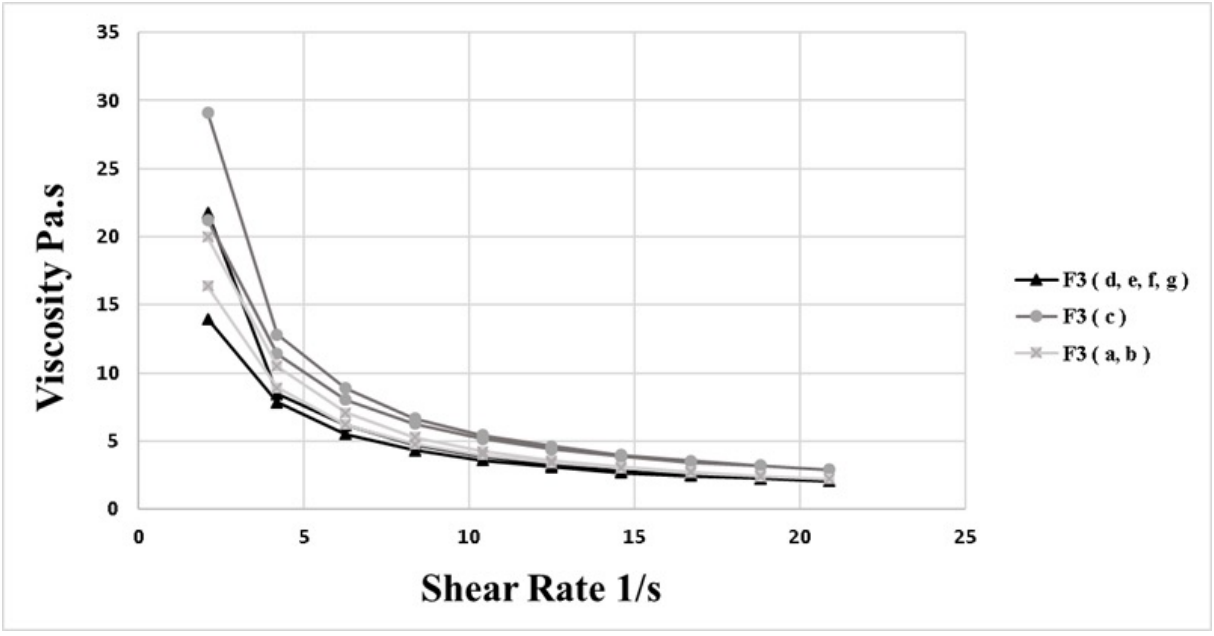

FIGURE 1 - Rheogram of formulations. F3 (d, e, f and g): Formulations with the combination of *R. mangle* extract and ascorbic acid; F3 (c): formulations with *R. mangle* extract; F3 (a and b): placebo of the formulations.

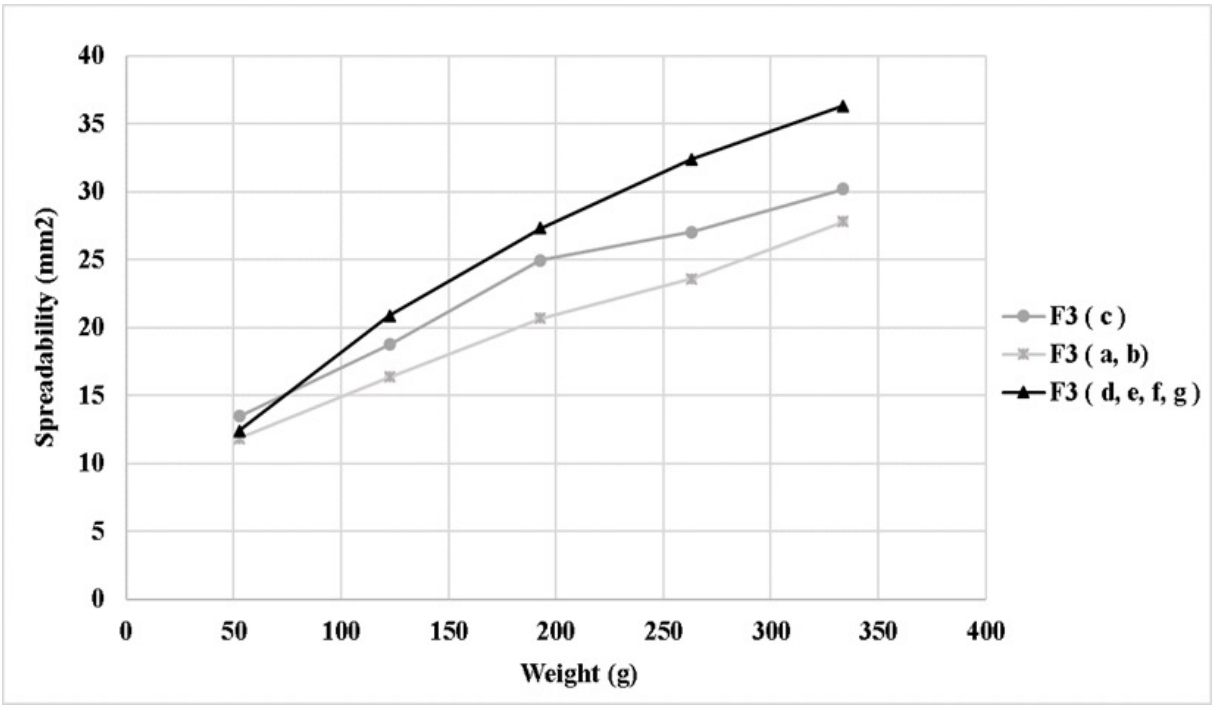

FIGURE 2 - Graphic of the spreadability of the formulations as a function of the weight (g) applied to the samples. F3 (d, e, f, g): Formulations with the combination of *R. mangle* extract and ascorbic acid; F3 (c): formulation with *R. mangle* extract F3 (a, b): placebo of the formulations.

Cytotoxicity Evaluation

Analysis of cell viability

After the pharmaceutical evaluation of the formulations (Table II), a cell viability assay was performed to evaluate whether the *R. mangle* and ascorbic acid hydrogels (i.e. active association) can damage animal cells. The cell viability test showed that 96% of the cells remained alive after treatment with the seven formulations analysed at concentrations of 0.05%, 0.1% and 0.2%. The concentration of 0.1% for all formulations was used in subsequent tests. The unviable cells were equivalent to 4%, and the F3 (d and f) formulations showed higher necrosis induction than the control, while formulation F3 (e) showed less apoptosis induction than the control. The apoptotic and necrotic values of cells treated with formulations F3 (a, b, c and g) were not statistically significant in relation to the control (Figure 3).

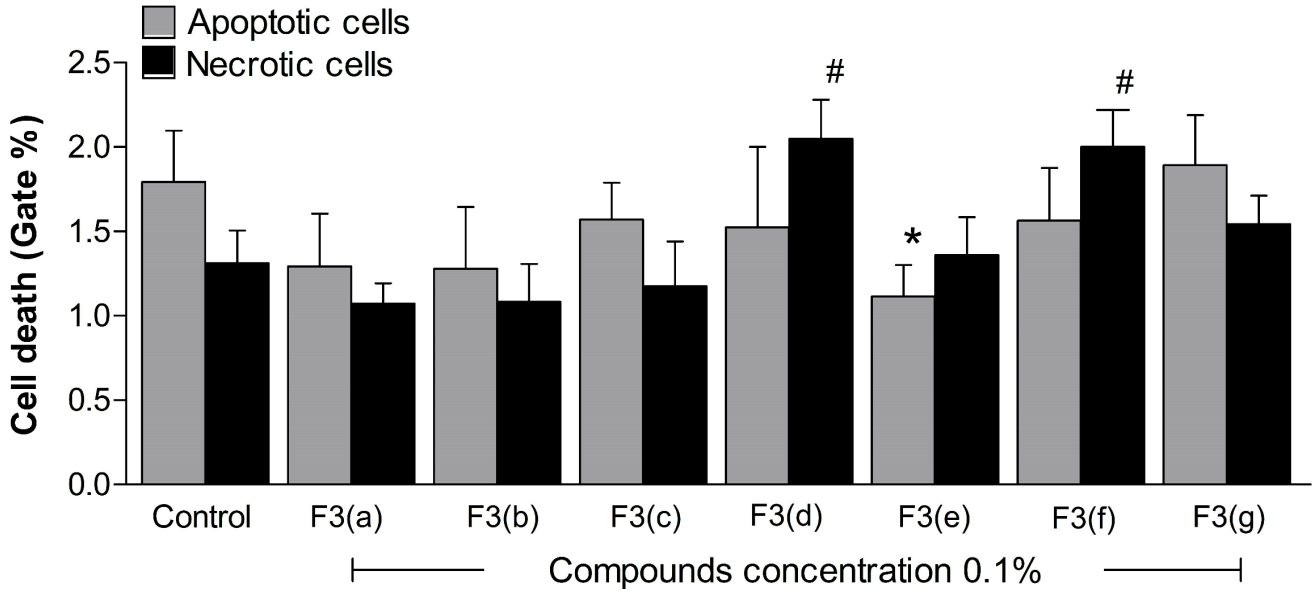

FIGURE 3 - Cell viability of the splenocytes of mice treated with formulations developed at different concentrations of the active association. Vertical grey bars represent the induction of cellular apoptosis; vertical black bars represent the induction of cellular necrosis. The vertical bars represent the average of the triplicate experiment. $^{\#}p < .0001$.

Cell proliferation

To assess whether the formulations, shown in Table II, were capable of activating mouse spleen immune cells, we conducted an assay to investigate their rate of proliferation during 2 days of cell culture. Lymphocytes did not show any significant proliferation compared with the control; however, monocytes showed a proliferation index when treated with formulations F3 (b, d and e) compared with the control (Figure 4).

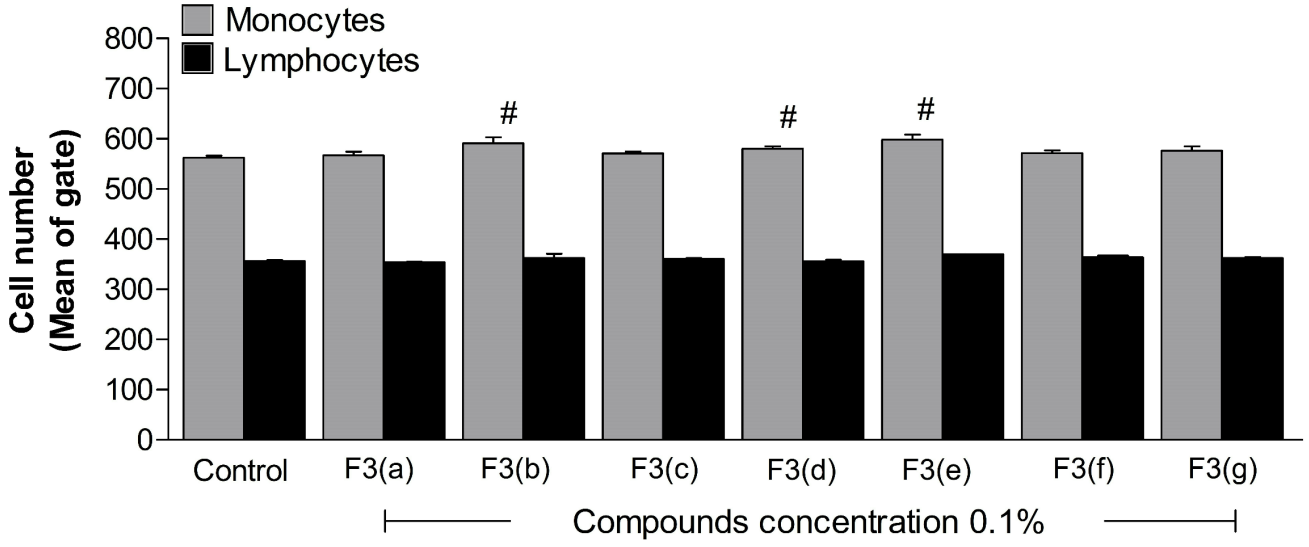

FIGURE 4 - Proliferation rate promoted by formulations developed in CFSE-labelled splenocyte cultures. Vertical grey bars represent treated monocyte cultures; vertical black bars represent treated lymphocyte cultures. The vertical bars represent the average of the triplicate experiment. $^{\#}p < .0001$.

Immunological Assays

Cytokine production

The investigation of cytokine production by lymphocytes and monocytes (i.e. IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and INF-γ) treated with hydrogels based on the association of *R. mangle* and ascorbic acid (Table II) did not reveal any production of IL-6 and INF-γ. However, the formulations F3 (c, d, e and f) induced the production of IL-2, IL-4, IL-10 and TNF-α, while the formulations F3 (c, d, e, f and g) induced the production of IL-17 compared with the control (Figure 5A, B, C, D and E). Thus, the F3 (e) showed the most significant induction of cytokine production by lymphocytes and monocytes of all formulations and the control.

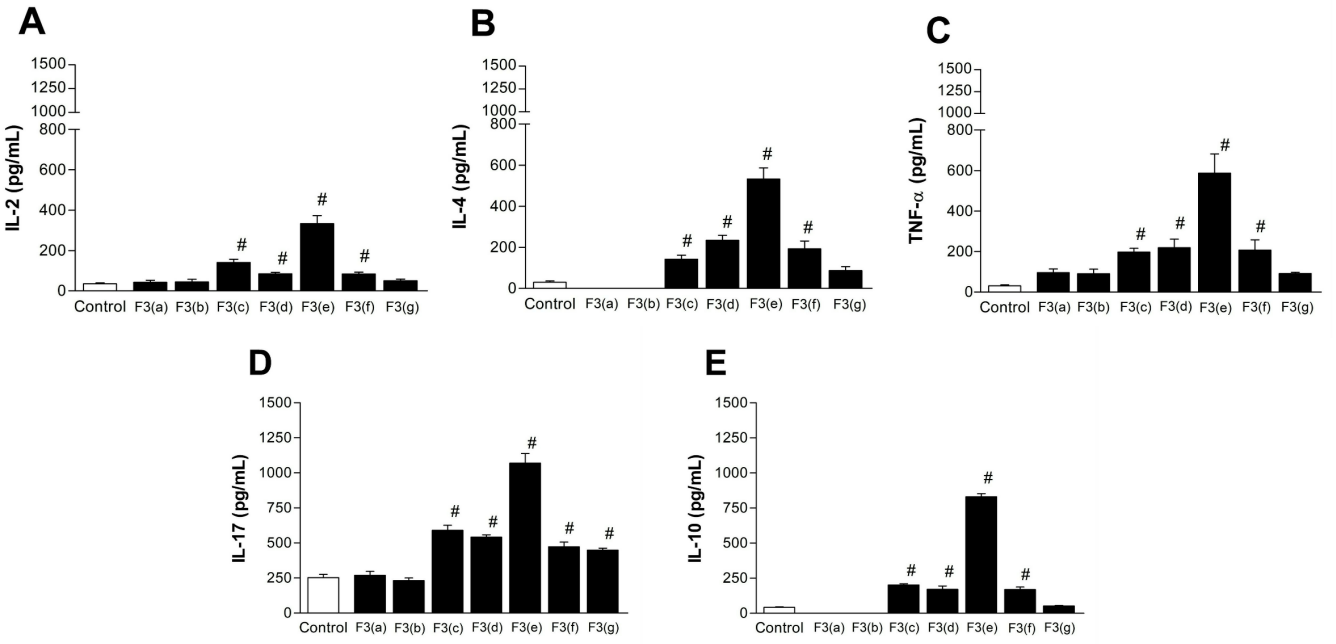

FIGURE 5 - Profile of IL-2, IL-4, IL-10, IL-17 and TNF-α cytokine production promoted by the seven formulations developed in mouse splenocyte cultures. The vertical white bars represent control cultures (i.e. cells plus averages); the vertical black bars represent cultures treated with the developed formulations. The vertical bars represent the average of the triplicate experiment. $^{\#}p < .0001$.

Lymphocyte immunophenotyping

Immunophenotyping assays were performed to analyse the ability of the formulations to induce the proliferation, activation and inhibition of $CD4^+$, $CD8^+$ and NK T lymphocyte sub-units (Table II). The results showed that the F3 (a) formulation induced the proliferation and significant activation of the $CD4^+$ T lymphocyte subgroup. F3 (b, c, d, f and g) did not induce any significant proliferation or activation, while F3 (e) induced the proliferation and activation inhibition of the $CD4^+$ T lymphocyte subgroup. Meanwhile, the formulations F3 (a, b, c, e, f and g) induced the proliferation and activation of the $CD8^+$ T lymphocyte subgroup compared with the control. The formulation F3 (d) did not show any effects on cells. Last, the F3 (b, c, d and f) formulations induced the proliferation and significant activation of the NK subgroup, while the F3 (a and g) formulations induced its inhibition (Figure 6).

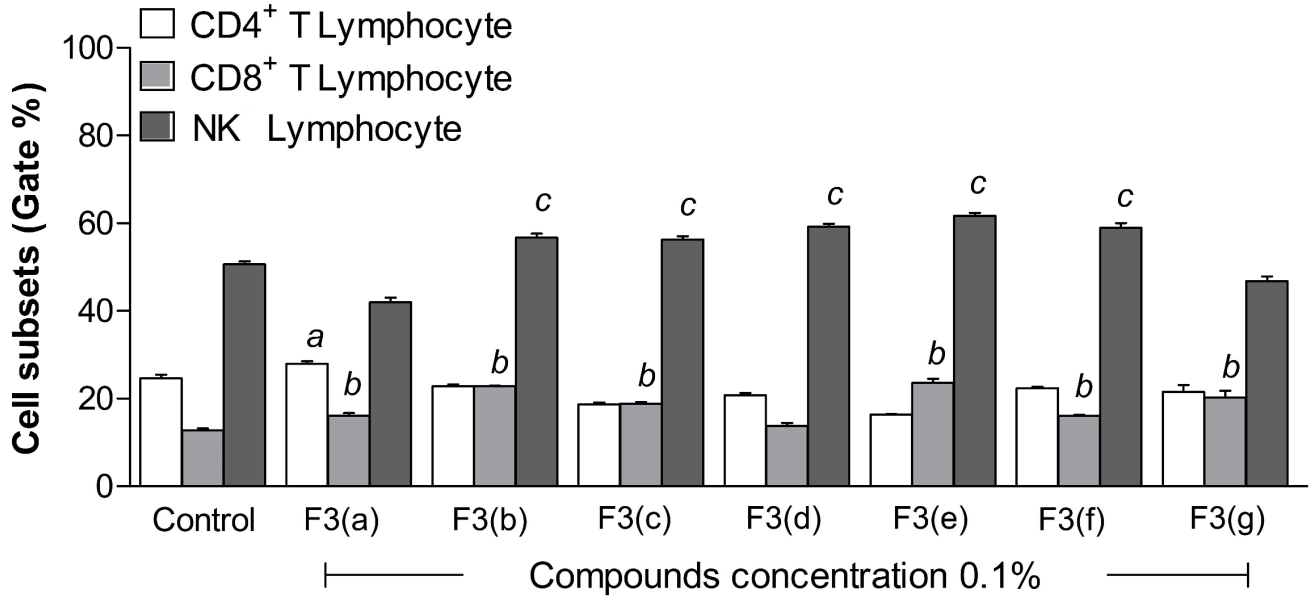

FIGURE 6 - *In vitro* proliferation, activation and inhibition assays induced by formulations based on the association of *R. mangle* extract and ascorbic acid in CD4 +, CD8 + and NK T lymphocyte subsets. The vertical bars represent the average of the triplicate experiment. # $p < .0001$.

DISCUSSION

The development of new materials for cell stimulation in wound healing and as immunostimulatory agents has become a field of wide scientific exploration. The use of herbal remedies such as *R. mangle* and products containing synthetic drugs such as ascorbic acid has shown promise in both health and biotechnology. Wounds are considered to be a public health problem in view of the numerous cases and especially given their high cost during treatment (Mata, Porto, Firmino, 2010). Therefore, a vast therapeutic arsenal composed of passive dressings and active principles is available for wound care (Smaniotto *et al*., 2012). However, few studies have examined the rational association of compounds proposed in this article.

The skin tolerates pH levels ranging from 4.6 to 5.8 (Gonçalves, Brianezi, Miot, 2017). The pH values of the seven formulations studied in our work ranged from 5.0 to 5.3 and are thus compatible with the pH values tolerated by the skin. In their study, Roger *et al*. (2011) revealed that the pH values of formulations containing *R. mangle* extract ranged from to 6.0 to 7.0; meanwhile, formulations with ascorbic acid have shown pH values ranging from 3.1 to 3.3 (Maia, 2002). The slightly acidic pH in the wound bed favours healing conditions and protection against infections and thus shows better results than with basic pH. It is also conducive to the penetration of ascorbic acid through the skin, which absorbs active better in more acidic media (Maia, 2002). In that context, Rubira *et al.* (2009) have highlighted that variation in temperature and pH level are linked to the degree of hydrogel swellings with changes in their morphological constitution. At the same time, Sixiang *et al.* (2018) have also described that varying pH values have different drug release profiles, such that alkaline environments show faster release than acidic ones.

Rheological characteristics are important properties to consider in the manufacture and application of topical products (Corrêa *et al*., 2005). In past research, hydrogels containing xanthan gum 1.5% showed non-Newtonian pseudoplastic behaviour, and their apparent viscosity gradually decreased as shear stress increased (Miura, 2012). Thus, rheology is an important feature of

topical formulations. According to Lourenço (2013), the absorption of active agents through the skin decreases with increased vehicle viscosity, which suggests an inverse relationship between viscosity and absorption. Another advantage of that behaviour is the ability to deform during application, which facilitates spreadability, followed by the recovery of viscosity when the application ends, which prevents the product from dripping (Corrêa *et al*., 2005).

The scattering characteristics of formulations on the skin are also important, both sensorially and in effect. Such importance corroborates rheological results when referring to the viscosity–spreadability relationship, namely that a decrease in viscosity implies an increase in spreadability (Andrade, 2017). The formulations evaluated in our study showed increased spreadability when a weight was used in them, which thus emerged as a favourable characteristic in relation to the topical formulation. In a study conducted with professionals from a wound outpatient clinic, Miura (2012) explored perspectives on the consistency of a material intended for application to wounds during dressing. The study revealed that the best formulation should be easy to apply and remove but not so fluid that it may slip from the boundaries of the lesion during the active period, which corroborates the characteristics of the hydrogels that we evaluated.

Toxicology, when used to evaluated harmful effects arising from interactions of chemicals in the body, is paramount in developing new formulations (Araújo, 2015). In our study, immune cells treated with hydrogels showed 96% viability, whereas the formulations did not show any cytotoxic profile characterising induction in monocyte proliferation and lymphocyte stability compared with the control. Although the active association (i.e. *R. mangle* extract and ascorbic acid) is not described in the literature, each component has been observed separately. Almeida (2017) has reported that the methanolic extract of *R. mangle* leaves did not show any cytotoxic profile against immune cells and was able to induce the proliferation of splenocytes. The aqueous extract of *R. mangle* leaves was also evaluated by Araújo (2015), who revealed that HeLa cell proliferation consequently had a mitogenic effect. Studies on ascorbic acid have also shown its selective cytotoxicity. Data obtained from *in vitro* and *in vivo* studies have revealed that the substance causes damage to tumour cells but preserves normal cells, and that effect has been verified in several strains. Even so, that selective response depends on the incubation period and the concentration (Mamede *et al*., 2012).

Cytokines are released by different host cells to stimulate and regulate other cells through specific receptors that participate in the control of all immunologically relevant events, including cell activation, differentiation, maturation, proliferation and survival (Oliveira *et al*., 2011). The active association in hydrogels induced the production of cytokines IL-2, IL-4, IL-10, IL-17 and TNF-α by lymphocytes and monocytes compared with the control. Studies with natural compounds have shown the induction of the production of cytokines such as IL-2, IL-6, TNF-α and IFN-γ using the methanolic extract of *R. mangle* leaves (Almeida, 2017) and TNF-α, IL-6 and IL-10 using lignin isolated from treated animal cells (Filho *et al*., 2019), as well as the induced of TNF-α, IL-2, IL-10 and IFN-γ in lymphocytes treated with the aqueous extract of *Conocarpus erectus* leaves (Santos *et al*., 2018). Meanwhile, studies with ascorbic acid have revealed the induced of IL-1 and TNF-α in response to different proinflammatory stimuli and various cell types (Kraychete, Calasans, Valente, 2006). Cytokines such as IL-2 and TNF-α are associated with the proinflammatory immune response present in tissue damage (Oliveira *et al*., 2011). By contrast, cytokines IL-4 and IL-10 aid in synthesising plasma cell antibodies and activating eosinophil, in addition to participating in scar response and fibrotic processes (Cinsa, Gualberto, Lopes, 2013; Medeiros, Filho, 2016). Beyond that, IL-17 plays fundamental regulatory roles in host defence and inflammatory diseases, as well as in post-surgical procedures, trauma and infections (Jin, Dong, 2013).

The results of immunophenotyping assays showed the significant proliferation and activation of the $CD8^+$ and NK T lymphocyte subgroups compared with the control. Gupta *et al.* (2011) have reported that *Browelia serrata* promoted a significant increase in the spleen CD4 and CD8 T cell subgroups, while Santos *et al.* (2018) showed that the aqueous extract of *Conocarpus erectus* leaves induced $CD8^+$ T lymphocyte activation

and proliferation without altering the $CD4^+$T lymphocyte subgroups and did not inhibit the of $CD4^+$ T and $CD8^+$ T lymphocyte subgroups. In other work, the ethanolic extract of *Psoralea corylifolia* seeds increased tumour cells by activating NK cells (Nunes-Pinheiro *et al.*, 2003). However, studies involving immunophenotyping with ascorbic acid have yet to be conducted. The activation and proliferation of immunological cells is directly related to the immune response that will be employed in adverse situations faced by the organism, including tissue repair and inflammation (Santos *et al.*, 2018).

Of the seven formulations evaluated in our study, F3 (e) with an active association of *R. mangle* extract 5% and ascorbic acid 10% showed the most expressive results in terms of cell proliferation and immunomodulatory activity. Along similar lines, Araújo (2015) developed a healing cream of the aqueous extract of *R. mangle* leaves at a concentration of 5% that showed favourable results in tissue repair during *in vitro* and *in vivo* tests. The same 5% concentration was also used by Lopes *et al.* (2019) to evaluate a healing cream with *Avicennia schaueriana* extract.

The concentrations of ascorbic acid in pharmaceutical formulations range from 5% to 15%, with the most useful concentration being 10% (Bagatin, 2009). Studies have shown that the 20% concentration in formulations leads to the maximum absorption level of the active through the skin; however, for reasons not yet established, higher concentrations result in a decrease in tissue levels (Azulay *et al.*, 2003), while the 15% concentration applied daily within 5 days showed, after 3 days, a saturation of ascorbic acid concentration in the skin (Dalcin, Schaffazick, Guterres, 2003). Those results suggest that the biological potential of hydrogels based on the association of *R. mangle* and ascorbic acid has been understood based on studies using the association of those compounds. However, it is necessary to expand research regarding the long-term conservation of components considering the sensitivity of the compounds in terms of luminosity and the oxidation control that can occur both with *R. mangle* extract and ascorbic acid. Such knowledge can be applied in future technical studies involving nanotechnology, which are expanding widely in scientific fields.

CONCLUSION

Xanthan gum 1.5% (F3) was chosen as the basis for hydrogels due to its compatibility with the active association studied (i.e. *R. mangle* and ascorbic acid) and characteristics such as homogeneous appearance, orange colour, smooth surface, intense gloss, an odour typical of the chemical compost a pH tolerable by skin and non-Newtonian pseudoplastic behaviour. The cell viability assay showed that formulations based on the association of *R. mangle* and ascorbic acid did not promote significant necrosis or apoptosis but could promote the proliferation of $CD4^+$, $CD8^+$ and NK T lymphocyte sub-units and immunomodulation in mouse splenocytes through the production of IL-2, IL-4, IL-10, IL-17 and TNF-α cytokines. Of the seven formulations evaluated, F3 (e) provided an initial understanding of the immune response, and those results may guide future studies using hydrogel based on xanthan gum in association with *R. mangle* extract and ascorbic acid as a potential cell stimulant agent used for wound healing and immunostimulatory evaluations.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

Aguiar LMS, Nascimento DKD, Ramos BA, Moura MC, Coelho LCBB, Napoleão TH, et al. Antioxidant, antimicrobial and immunostimulant properties of saline extract from *Caesalpinia pulcherrima* (L.) Swartz (Fabaceae) leaves. Indian J Tradit Knowl. 2019;18(2):281-89.

Almeida VS. Investigação de atividade citotoxica, antibacteriana e imunomoduladoura do extrato metanolico de *Rhizophora mangle Linnaeus* (Rhizophoraceae). [Dissertation]. Recife: Universidade Federal de Pernambuco. 2017.

Andrade VM. Obtenção de gel plo contendo rutina para aplicação transdérmica: caracterização, estabilidade e atividade antioxidante. [Dissertation]. São Cristóvão: Universidade Federal de Sergipe. 2017.

Araújo JG. Desenvolvimento de creme de *Rhizophora mangle* L: avaliação do potencial cicatrizante em feridas cutâneas. [Dissertation]. Recife: Universidade Federal de Pernambuco. 2015.

Azulay MM, Lacerda CAM, Perez MA, Filgueira AL, Cuzzi T. Vitamina C. An Bras Dermat. 2003;78(3):265-74.

Bagatin E. Mecanismos do envelhecimento cutâneo e o papel dos cosmecêuticos. Rev Bras Med. 2009;66(3):5-11.

Brasil. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Guia de estabilidade de Produtos Cosméticos. Série Temáticas; Brasília: ANVISA; 2004. Disponível em: http://portal.anvisa.gov.br/documents/106351/107910/Guia+de+Estabilidade+de+Produtos+Cosm%C3%A9ticos/49cdf34c-b697-4af3-8647-dcb600f753e2. Acesso em: 11/09/2019.

Biaou OOB, Lallepak L, Bianza MB, Saied AH, Guang Y. Combining silk sericin and surface micropatterns in bacterial cellulose dressings to control fibrosis and enhance wound healing. Eng Sci. 2020;10:68-77.

Borghetti GS, Knorst MT. Desenvolvimento e avaliação da estabilidade física de loções O/A contendo filtros solares. Rev Bras Ciên Farm. 2006;42(4):531-7.

Campos ACL, Borges-Branco A, Groth AK. Cicatrização de feridas. Arq Bras Cir Dig. 2007;20(1):51-58.

Cinsa L, Gualberto ACM, Lopes KHS. Processo cicatricial cutâneo e perfil de citocinas. Rev Interdisciplin Estud Exp. 2013;5(único):17-21.

Corrêa NM, Camargo JFB, Ignácio RF, Leonardi GR. Avaliação do comportamento reológico de diferentes géis hidrofílicos. Rev Bras Farm. 2005;41(1):73-78.

Dalcin KB, Schaffazick SR, Guterres SS. Vitamina C e seus derivados em produtos dermatológicos: Aplicações e Estabilidade. Cad Farm. 2003;19(2):69-79.

Dumortier G, Grossiord JL, Agnel YF, Chaumeil JC. A review of poloxamer 407 pharmaceutical and pharmacological characteristics. Pharm Res. 2006;23(12):2709-28.

Filho I JC, Barros BRS, Aguiar LMS, Navarro CDC, Ruas JS, Lorena VMB, et al. Lignins isolated from prickly pear cladodes of the species *Opuntia ficusindica* (Linnaeus) Miller and *Opuntia cochenillifera* (Linnaeus) Miller induces mice splenocytes activation, proliferation and cytokines production. Int J Bio Macromol. 2019;15:1331-39.

Goebel K. Estudo de liberação in vitro do Diclofenaco dietilamônio em especialidades farmacêuticas. [Dissertation]. Curitiba: Universidade Federal do Paraná. 2012.

Gonçalves GM, Brianezi G, Miot HA. O pH dos principais hidratantes e sabonetes líquidos comerciais brasileiros: considerações sobre o reparo da barreira cutânea. Na Bras Dermatol. 2017;92(5):738-40.

Gupta A, Khajuria A, Singh J, Singh KA, Suri GN. Immunological adjuvant effect of *Boswellia serrata* (BOS 2000) on specific antibody and cellular response to ovalbumin in mice. Int Immunopharmacol. 2011;11(8):968-75.

Jin W, Dong C. IL-17 cytokines in immunity and inflammation. Emerg Microbes Infect. 2013;2(9):52-60.

Kraychete DC, Calasans MTA, Valente, CML. Citocinas pró-inflamatórias e dor. Rev Bras Reumatol. 2006;46(3):199-206.

Lopes CMI, Bararella-Evêncio L, Souza IA, Oliveira E, Sá JGA, Santana MAN, et al. Evaluation of cytotoxicity and wound healing activity of *Avcennia schaueriana* in cream. An Acad Bras Ciênc. 2019;91(1):20280171-13.

Lourenço ARN. Administração Tópica de Fármacos - das Restrições aos Desafios. [Dissertation]. Lisboa: Universidade Lusófona de Humanidades e Tecnologias. 2013.

Maia MA. Desenvolvimento e avaliação da estabilidade das formulações cosméticas contendo ácido ascórbico. [Dissertation]. São Paulo: Universidade de São Paulo. 2002.

Mamede AC, Pires AS, Abrantes AM, Tavares SD, Gonçalves AC, Casalta-Lopes JE, et al. Cytotoxicity of ascorbic acid in a human colorectal adenocarcinoma cell line (WiDr): in vitro and in vivo studies. Nutr Cancer. 2012;64(7):1049-57.

Marrero E, Sánchez J, Armas E, Escobar A, Melchor G, Abad MJ, et al. COX-2 and sPLA2 inhibitory activity of aqueous extract and polyphenols of *Rhizophora mangle* (red mangrove). Fitoterapia. 2006;77(4):313-15.

Mata VE, Porto F, Firmino F. Tempo e custo do procedimento: curativo em úlcera Vasculogênica. Rev Pesqui Cuid Fundam. 2010;2(Ed.Supl.):94-7.

Mazhar U, Jawad A, Waliullah K, Adnan H, Nasrullah S, Md. WA, et al. Fast 4-nitrophenol reduction using gelatin hydrogel containing silver nanoparticles. Eng Sci. 2019;8:19-24.

Medeiros AC, Filho AMD. Cicatrização das feridas cirúrgicas. J Surg Clin Res. 2016;7(2):87-102.

Miura DY. Desenvolvimento farmacotécnico e estudo de estabilidade de géis de papaína destinados ao tratamento

de feridas. [Dissertation]. Niterói: Universidade Federal Fluminense. 2012.

Mohsin J, Shaikh JRH. Preparation and evaluation of herbal gel formulation. J Pharm Res Educ. 2017;1(2):201-24.

Nunes-Pinheiro DCS, Leite AKRM, Farias VM, Braga LT, Lopes CAP. Atividade imunomoduladora das plantas medicinais: perspectivas em medicina veterinária. Cienc Anim Bras. 2003;13(1):23-32.

Ofori-kwakye K, Kwapong AA, Bayor MT. Wound healing potential of methanol extract of *Spathodea campanulata* stem bark formulated into a topical preparation. Afr J Tradit Complement Altern Med. 2011;8(3):218-23.

Oliveira CMB, Sakata RK, Issy AM, Gerola LR, Salomão R. Citocinas e dor. Rev Bras Anestesiol. 2011;61(2):255-65.

Oliveira JAV. Aplicação da *Rhizophora mangle* associada ao biopolímero de betaglucana em retalhos cutâneos isquêmicos em ratos. [Dissertation]. Recife: Universidade Federal de Pernambuco. 2018.

Pessoa MFA. A administração sistêmica e tópica de vitaminas antioxidantes acelera a cicatrização de feridas cutâneas em camundongos diabéticos. [Thesis]. São Paulo: Universidade de São Paulo. 2014.

Prista LN, Alves AC, Morgado R, Lobo JS. Tecnologia Farmacêutica. 6a ed. Lisboa: Fundação Calouste Gulbenkian. 2008.

Regalado AI, Sánchez LM, Mancebo B. *Rhizophora mangle* L. (mangle rojo): Una especie con potencialidades de uso terapêutico. J Pharm Pharmacogn Res. 2016;4(1):1-17.

Roger DMS, Perdomo YR, Bueno TP, Alemán YR, Lacarrere IGM. Estabilidad acelerada de un gel de *Rhizophora mangle* L. (mangle rojo) para heridas y quemaduras. Rev Cubana Farm. 2011;45(5):563-74.

Rubira AF, Muniz EC, Guilherme MR., Paulino AT, Tambourgi EB. Morfologia de hidrogéis-ipn termo-sensíveis e ph-responsivos para aplicação como biomaterial na cultura de células. Polímeros. 2009;19(2):105-110.

Santos DKDN, Almeida VS, Araujo DRC, Harand W, Soares AKA, Moreira LR, et al. Evaluation of cytotoxic, immunomodulatory and antibacterial activities of aqueous extract from leaves of *Conocarpus erectus* Linnaeus (Combretaceae). J Pharm Pharmacol. 2018;70(8):1092-1101.

Silva MMP, Aguiar MIF, Rodrigues AB, Miranda MDC, Araújo MAM, Rolim ILTP, et al. The use of nanoparticles in wound treatment: a systematic review. Rev Esc Enferm USP. 2017;51:32-72.

Sixiang L, Ashwak J, Weiwei Z, Lina F, Muhammad WU, Zhijun S, et al. Fabrication of pH-electroactive bacterial cellulose/polyaniline hydrogel for the development of a controlled drug release system. ES Mater Manuf. 2018;1:41-49.

Smaniotto PHS, Castro FM, Cesar I, Rafael G. Sistematização de curativos para o tratamento clínico das feridas. Rev Bras Cir Plást. 2012;27(4):623-626.

Oliveira CMB, Sakata RK, Issy AM, Gerola LR, Salomão R. Cytokines and pain. Rev Bras Anestesiol. 2011; 61(2):260-5. https://doi.org/10.1590/S0034-70942011000200014

Xiaosai H**,** Rui L, Jun L, Zhengping L, Guoxing S. Highly stretchable self-healing nanocomposite hydrogel reinforced by 5 nm particles. ES Mater Manuf. 2018;2:16-23.

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