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JANAÍNA ARTEM ATAIDE

DESENVOLVIMENTO DE PARTÍCULAS POLIMÉRICAS COMO SISTEMAS CARREADORES DE PRODUTO BIOATIVO

DEVELOPMENT OF POLYMERIC PARTICLES AS CARRIER SYSTEMS FOR BIOACTIVE PRODUCTS

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Dissertação apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Ciências, Área de Concentração Pesquisa Clínica.

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"Para ser grande, sê inteiro: nada
Teu exagera ou excluí.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive."

Põe quanto és no mínimo que fazes
(Ricardo Reis)

À vó Bida e a vó Rosa (*in memoriam*)

"You think the dead we have loved ever truly leave us? You think that we don't recall them more clearly than ever in times of great trouble?"

(J. K. Rowling em *"Harry Potter and the Prisoner of Azkaban"*)

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RESUMO

A bromelina, um complexo de substâncias extraídas principalmente do abacaxi (*Ananas comosus* L.), apresenta reconhecidas propriedades anti-inflamatória, antitrombótica e fibrinolítica, atividade antitumoral e efeito imunomodulador, o que tem atraído atenção para seu uso no cuidado da pele. No entanto, a bromelina é geralmente instável em condições de estresse, o que resulta em uma diminuição de sua atividade enzimática, limitando suas aplicações farmacológicas e industriais. A nanoencapsulação da bromelina pode aumentar sua estabilidade, eficácia e segurança, além de modificar sua cinética de liberação. A quitosana, um polímero natural, forma nanoestruturas que podem aprisionar a enzima, mantendo as reivindicações de biocompatibilidade, biodegradabilidade e fonte natural da formulação como todo. Sendo assim, o presente trabalho teve como objetivo encapsular a bromelina em nanopartículas de quitosana, a fim de aumentar a estabilidade desse complexo enzimático. Para isso, nanopartículas de quitosana-bromelina foram produzidas pelo método de geleificação iônica, resultando em partículas esféricas com 100,9 ± 0,5 nm e índice de polidispersão de 0,222 ± 0,012. A eficiência da encapsulação foi de 87,4% da concentração de proteínas, correspondendo a 80,7% da atividade enzimática. No entanto, a nanopartícula de quitosana-bromelina não apresentou a estabilidade desejada quando armazenada em suspensão aquosa, e por isso foram liofilizadas. Glicina ou maltose foram utilizadas como lioprotetores, resultando em um produto elegante e com curto tempo de reconstituição, alterando o tamanho das nanopartículas e aumentando a taxa de encapsulação quando comparada a forma líquida. Além disso, o trabalho também tinha como objetivo avaliar a atividade e a toxicidade *in vitro* das nanopartículas produzidas. Para isso, ensaios de atividades antioxidante e antioproliferativa foram conduzidos com a bromelina livre e nanoencapsulada. Após o processo de nanoencapsulação, a bromelina manteve sua atividade antioxidante e antiproliferativa, no entanto foram necessários maior tempo ou maior concentração para o mesmo efeito ser observado, sugerindo que a bromelina foi encapsulada de forma eficiente e que sua cinética de liberação pode ter sido modificada. Outro teste de atividade conduzido foi o teste de *scratch in vitro* com queratinócitos, no qual a nanoencapsulação da bromelina com a quitosana também se mostrou efetiva em aumentar a retração de ferida no ensaio de *scratch in vitro* com queratinócitos, quando comparada a bromelina livre. Os resultados obtidos nos permitem concluir que a bromelina foi encapsulada nas nanopartículas de quitosana com eficiência satisfatória. A formulação final foi liofilizada com a adição de glicina ou maltose como

lioprotetores, resultando em uma maior eficiência de encapsulação da bromelina, assim como uma maior estabilidade. Após a encapsulação, a bromelina manteve sua atividade antioxidante e antiproliferativa, sendo dependente do uso de uma maior concentração. Além disso, a nanoencapsulação aumentou sua atividade na retração de ferida no ensaio de *scratch in vitro*.

Palavras-chave: bromelina; nanopartículas; quitosana; estabilidade de medicamentos; liofilização

ABSTRACT

Bromelain, a complex of substances extracted mainly from pineapple (*Ananas comosus* L.), has recognized properties such as anti-inflammatory, antithrombotic and fibrinolytic effects, antitumor activity and immunomodulatory effect, which has attracted attention for its use in skin care. However, bromelain is usually unstable under stress conditions, which results in a decrease of enzymatic activity and limits its pharmacological and industrial applications. Encapsulation of bromelain in nanoparticles can increase its stability, efficacy and safety, besides modification of its release kinetics. The natural polymer chitosan forms nanostructures that can entrap the enzyme, maintaining the claims of biocompatibility, biodegradability and natural source of the whole formulation. Therefore, the present work had as objective to encapsulate bromelain in chitosan nanoparticles in order to increase the stability of this enzymatic complex. For this, chitosan-bromelain nanoparticles were produced by ionic crosslinking, resulting in spherical particles with 100.9 ± 0.5 nm and polydispersity index of 0.222 ± 0.012. Encapsulation efficiency was 87.4% of total protein concentration, corresponding to 80.7% of enzymatic activity. However, chitosan-bromelain nanoparticles did not show the desired stability when stored in aqueous suspension, and were lyophilized. Glycine or maltose were used as lyoprotectants, resulting in an elegant product with a short reconstitution time, altering nanoparticles size and increasing the encapsulation rate when compared to the liquid form. In addition, this work also aimed to evaluate the activity and *in vitro* toxicity of the nanoparticles produced. For this, antioxidant and antiproliferative activity assays were conducted with free and nanoencapsulated bromelain. After nanoencapsulation process, bromelain maintained its antioxidant and antiproliferative activity, however, a greater time or concentration were required so the same effect could be observed, suggesting that bromelain was efficiently encapsulated and that its release kinetics may have been modified. Another activity test conducted was the *in vitro* scratch test with keratinocytes, in which nanoencapsulation of bromelain with chitosan was also effective in increasing wound retraction in *in vitro* scratch assay with keratinocytes when compared to free bromelain. Obtained results obtained allow us to conclude that bromelain was encapsulated in chitosan nanoparticles with satisfactory efficiency. The final formulation was lyophilized with the addition of glycine or maltose as lyoprotectants, resulting in enhanced bromelain encapsulation efficiency, as well as greater stability. After encapsulation, bromelain maintained its antioxidant and antiproliferative

activity, being dependent on a higher concentration. In addition, nanoencapsulation increased its activity in wound retraction in *in vitro* scratch assay.

Keywords: bromelain; nanoparticles; chitosan; drug stability; freeze-drying

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LISTA DE ABREVIATURAS E SIGLAS

% = porcentagem

° C = graus Celsius

ABTS = 2,2-azino-bis-3-ethylbenzothiazoline-6 sulfonic acid

ANVISA = agência nacional de vigilância sanitária

BET = brunauer–Emmett–Teller

BJH = barrett–Joyner–Halenda

CLA = lactobionic acid-modified chitosan

CMCS = modified carboxymethyl chitosan

DLS = dynamic light scattering

DPPH = 2,2-diphenyl-1-picrylhydrazyl

GI_{50} = 50% of growth inhibition

h = hora

HepG2 = human liver carcinoma cell line

kDa = quilodalton

LCN = lipid-core nanocapsule

MBC = minimum bactericidal concentration

MCF-7 = human breast cancer cell line

MEV = microscopia eletrônica de varredura

mg = miligrama

MIC = minimum inhibitory concentration

min = minuto

mL = mililitro

nm = nanometro

NP = nanoparticle

NTA = nanoparticle tracking analysis

PAA = poly(acrylic acid)

PBS = phosphate buffer solution

PCL = poly(ε-caprolactone)

PDI = polydispersity index

pI = isoelectric point

PLGA = poly(lactide-co-glycolide) acid

rpm = rotações por minuto

SEM = scanning electron microscopy

SH-SY5Y = human neuroblastoma cancer cell line

TEM = transmission electron microscopy

TGI = total growth inhibition

TPP = tripolifosfato de sódio

μL = microlitro

μm = micrometro

LAC = chitosan oligossacharide lactate

LMW = low molecular weight chitosan

SHR = chitosan from shrimp shells

FTIR = Fourier transform infrared

RCF = relative centrifugal force

SD = standard deviation

Z-ave = mean nanoparticle diameter

D_{10} = percentile 10

D_{50} = percentile 50

D_{90} = percentile 90

m/v = massa/volume

w/v = weight volume

w/w = weight/weight

SUMÁRIO

1. INTRODUÇÃO

Bromelina é um nome coletivo para enzimas proteolíticas ou proteases encontradas em tecidos do abacaxi (*Ananas comosus*) e de diversas espécies da família Bromeliaceae (Hennrich *et al.*, 1969; Taussig and Batkin, 1988; Doko *et al.*, 1991; Maurer, 2001; Fitzhugh *et al.*, 2008; Chobotova *et al.*, 2010; Hebbar *et al.*, 2012; Chaurasiya and Umesh Hebbar, 2013). Devido à sua atividade proteolítica, a bromelina tem aplicações potenciais nas indústrias cosmética, farmacêutica e de alimentos. A partir do estudo de Seligman que mostrou, em 1962, sua ação como agente antiinflamatório, vários estudos sustentam o uso de extratos de bromelina em diferentes condições (Seligman, 1962; Taussig and Batkin, 1988; Salas *et al.*, 2008; Chobotova *et al.*, 2010; Amid *et al.*, 2011; Ferreira *et al.*, 2011).

Em particular, alguns estudos tem demonstrado o potencial uso da bromelina em processos de cicatrização. Maurer (2001) demonstrou que essa enzima possui benefícios para a cicatrização de feridas, especificamente reduzindo edema, hematomas e dor. Em queimaduras, a bromelina age hidrolisando o tecido desvitalizado, tanto *in vivo* quanto *in vitro*, o que aumenta a capacidade de cicatrização. Processos inflamatórios também estão envolvidos na cicatrização, além da oxigenação do tecido em feridas de queimadura. A melhora dessa oxigenação é uma chave para a total cicatrização e a bromelina pode ser uma forma de aprimorá-la, sendo que essa hipótese deve ser melhor elucidada (Wu *et al.*, 2012). Singer *et al.* (2010) já haviam demonstrado que uma única aplicação tópica de uma preparação contendo bromelina sobre queimaduras em pele de porco, das quais a queratina necrótica havia sido removida, resultou em um rápido debridamento e dissolução da derme necrótica.

O uso de proteínas como ingrediente ativo em produtos farmacêuticos é um desafio, devido à sua instabilidade física e química. Os processos de instabilidade podem levar à redução ou à perda da atividade biológica, ou até mesmo alterar seu potencial imunogênico (Sanchez-Ruiz and Makhatadze, 2001). A bromelina cliva, preferencialmente, ligações peptídicas glicil, alanil e leucil (Maurer, 2001). Seu mecanismo catalítico envolve a oxidação de grupos sulfidrilas (-SH), levando a formação de pontes dissulfeto. Este processo conduz a uma clivagem autoproteolítica, reduzindo assim a atividade enzimática da bromelina (Bala *et al.*, 2012). Pereira *et al.* (2014) demonstraram que formulações com bromelina, quando conservadas a 37 °C, perdiam quase toda sua atividade enzimática. Eles atribuíram este fato à auto-degradação (autólise ou autodigestão) da bromelina, pois 37 °C tem sido considerada a temperatura ótima para sua atividade proteolítica (Pereira *et al.*, 2014).

Nos últimos anos, o desenvolvimento de sistemas nanoparticulados biodegradáveis para a liberação de fármacos vem despertando grande interesse. Esses carreadores coloidais apresentam diversas vantagens, tais como possibilidade de proteção do ativo encapsulado frente à degradações *in vivo*, relativa estabilidade nos fluidos biológicos e a capacidade de modulação da liberação do fármaco e, por isso, são considerados bastante promissores (Lemarchand *et al.*, 2004; Le Droumaguet *et al.*, 2012). Em geral, os polímeros utilizados no preparo das nanopartículas apresentam características adequadas no que diz respeito à biocompatibilidade, biodegradabilidade e toxicidade. Além disso, podem proteger o composto bioativo frente à degradação e controlar sua liberação (Lopes *et al.*, 2010).

Devido a suas características de adesividade, biocompatibilidade, biodegradabilidade e baixa toxicidade, a quitosana tem se tornado um material potencialmente atraente para diversos usos, principalmente na área farmacêutica. Este polissacarídeo vem sendo usado como sistema polimérico na liberação modificada de fármacos de diversas classes terapêuticas, tais como, antibióticos, antiinflamatórios, anti-hipertensivos, além de peptídeos e proteínas (Bernkop-Schnürch, 2000; Florea *et al.*, 2006; Boonyo *et al.*, 2007; Sandri *et al.*, 2010). Além disso, o uso da quitosana para o tratamento de feridas e queimaduras tem sido estudado, com base em sua capacidade hemostática e seu efeito de aceleração no reparo de feridas com melhor efeito estático final. A facilidade de adesão da quitosana, assim como seu caráter antifúngico, bacteriostático e sua permeabilidade ao oxigênio são propriedades muito atrativas para uso tópico (Argüelles, 2004; Jayakumar *et al.*, 2011).

Ainda existem poucas referências na literatura sobre o encapsulamento da bromelina como ativo principal da formulação e sobre sua estabilidade após encapsulada. Dessa forma, o presente trabalho pretende a incorporação de bromelina em nanopartículas de quitosana, a fim de aumentar a estabilidade e controlar a liberação dessa enzima, visando sua utilização terapêutica no tratamento de feridas. Sendo assim, o presente trabalho encontra-se dividido em capítulos, regidos sobre forma de artigos, a saber:

Capítulo I. “Wound Healing Process and Synthetic Actives: a Review”

Capítulo II. “Natural Actives for Wound Healing: a Review”

Capítulo III. “Bromelain-Loaded Nanoparticles: a Comprehensive Review of the State of the Art”

Capítulo IV. “Bromelain-Chitosan Nanoparticles: Effect of Polysaccharide Sources on the Physicochemical Properties of Nanoparticles”

Capítulo V. “Freeze-Dried Chitosan Nanoparticles to Stabilize and Deliver Bromelain”

Capítulo VI. “Antioxidant and Antiproliferative Activities of Free and Chitosan-Encapsulated Bromelain”

Desta forma, determinadas informações que constam em um capítulo poderão ser repetidas nos capítulos seguintes, assim como na discussão e na conclusão. As referências encontram-se ao final de cada capítulo, formatadas de acordo com as normas das revistas científicas em que os artigos foram/serão submetidos. Ao final do trabalho, há lista de referências da introdução e discussão.

2. OBJETIVOS

2.1. Objetivo Geral

Encapsular a bromelina comercialmente disponível em nanopartículas de quitosana.

2.2. Objetivos Específicos

- Avaliar a estabilidade da bromelina frente à variação de pH, de temperatura e de polímeros presente no meio;
- Desenvolver as nanopartículas de quitosana-bromelina;
- Caracterizar físico-química e morfologicamente as nanopartículas de quitosana-bromelina;
- Avaliar a eficiência de encapsulação da bromelina;
- Avaliar a liberação da bromelina a partir das nanopartículas de quitosana-bromelina;
- Avaliar a estabilidade das nanopartículas de quitosana-bromelina;
- Avaliar o processo de liofilização das nanopartículas de quitosana-bromelina;
- Avaliar a atividade antioxidante e antiproliferativa das nanopartículas de quitosana-bromelina *in vitro*.

3. EXECUÇÃO

CAPÍTULO I. "WOUND HEALING PROCESS AND SYNTHETIC ACTIVES: A REVIEW"

Janaína Artem Ataide, Letícia Caramori Cefali, Lucas Militão, Beatriz Zanchetta, Eliana B. Souto, Laura Oliveira-Nascimento, Priscila Gava Mazzola

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Abstract

Wound healing is a known complicate and intricate process. It is essential that healthcare professionals understand the key process involved in the healing cascade, to maximize care with these patients and minimize the undesirable outcomes of non-healing wounds. It is also important to review and highlight the common used dressings and synthetic active, helping healthcare professionals in wound management. Thus, this review aims to summarize the healing process phases, and the used wound dressings and actives.

Key words: dressings; healing; synthetic actives; wound

Introduction

Disruption of the integrity of skin, mucosal surfaces or organ tissue results in the formation of a wound (Young and McNaught, 2011), and although skin injuries vary, they have a common innate mechanism for repair and healing. Wound healing is a regular biological process in the human body, once human skin has a natural ability to promote self-regeneration after damage (Guo and DiPietro, 2010; Pereira and Bártolo, 2016), and when triggered by an injury, this mechanism comprises an elaborate cascade of physiologic events designed to repair and ultimately heal the skin (Strodtbeck, 2001).

However, this body capacity is dependent on many known factors, such as patient's underlying health and nutritional status (Beldon, 2010), and can be compromised under specific conditions, like diabetes, non-healing ulcers, extensive skin loss, and deep burns (Groeber et al., 2011; Guo and DiPietro, 2010). Given the intricate nature of the healing cascade, it is remarkable how often healing occurs without complication (Young and McNaught, 2011). An inappropriate healing process can lead the wound to enter in a chronic state, which increases the risk of infection and affects patient's health and quality of life (Pereira and Bártolo, 2016), with potential associated morbidity and mortality, and poor cosmetic outcome (Abdelrahman and Newton, 2011; Young and McNaught, 2011).

Although it is difficult to quantify the economic effects of chronic wounds, it has been estimated that the annual expenditure on wound related problems in the USA alone exceeds one billion dollars (Ueno et al., 2006; Young and McNaught, 2011). It is therefore essential that healthcare professionals understand the key physiological processes involved in healing in order to minimize patient morbidity from delayed healing (Young and McNaught, 2011). This review aims to summarize relevant and overlapping phases of the healing process, wound dressings and synthetic actives currently used during wound management, aiming to help healthcare professionals in the wound management.

1. Wounds and Healing

According to the Wound Healing Society, a wound is the "disruption of normal anatomic structure and function" (Lazarus et al., 1994), that can be classified as acute or chronic. Acute wounds are typically tissue injuries that heals within the expected period. In contrast, chronic wounds are tissue injuries that heal sluggishly because of repeated tissue damages and/or other underlying pathophysiology that interferes with the expected time line or wound healing sequence (Lazarus et al., 1994; Strodtbeck, 2001).

Healing initiates in response to an injury, to restore function and integrity of the damaged tissue and consequently homeostasis (Gottrup et al., 2000; Gurtner et al., 2008; Martin, 1997; Reinke and Sorg, 2012; Schmidt et al., 2009). Wound Healing Society defines it as "a complex dynamic process that results in the restoration of anatomic continuity and function" (Lazarus et al., 1994). The normal process comprehend four overlapping phases (Fonder et al., 2008; Kumar et al., 2007; Schmidt et al., 2009; Strodtbeck, 2001), regulated by cellular, humoral and molecular mechanisms (Reinke and Sorg, 2012).

The first stage, haemostasis, occurs immediately at the time of injury and usually lasts a few hours. The second stage, inflammation, begins shortly after haemostasis and is usually completed within the first 24 to 72 hours after injury; however, it may last up to 5-7 days after injury (Haas, 1995). Proliferation and repair, the third stage, typically occurs 1 to 3 weeks after injury. The fourth and final stage, remodelling, begins approximately 3 weeks after injury and may take months to several years to achieve physiological completion. Therefore, it is important to note that although the skin seems intact, the tissue underneath is still vulnerable to damage as it undergoes the final stages of wound healing (Bryant and Nix, 2016; Reinke and Sorg, 2012; Strodtbeck, 2001).

1.1. Haemostasis and coagulation

Many authors describe haemostasis and inflammation as a unique phase, mainly because these conditions are closely linked since haemostasis and coagulation initiate the inflammatory process. For the sake of clarity, in this review these phases will be discussed separately. With a skin injury outreaching the epidermal layer, blood and lymphatic vessels are traumatized, flushing the wound to remove microorganisms and antigens (Strodtbeck, 2001). The first stage of physiological or acute wound healing is dedicated to haemostasis and the formation of a provisional wound matrix, which occurs immediately after injury and is completed after some hours (Reinke and Sorg, 2012).

Generally, there are three main haemostatic mechanisms engaged in reducing blood loss: vascular spasm, platelet plug formation and coagulation. Vascular spasm, also called vasoconstriction, is the initial response to the injury that induces systole of the smooth muscle in the walls of the vessels (Bhagavan and Ha, 2011). Then, exposed collagen by vessel rupture aid in platelet aggregation and adhesion to its surface; platelet plug seals small vessel injuries and releases substances that maintain vasoconstriction, activate more free platelets to adhere and signalizes the beginning of the clot cascade.

The crucial process in haemostasis is blood clotting, which is the transformation of blood state from liquid to solid with the assistance of clotting factors (Jin and Gopinath, 2016). Thrombin activates fibrinogen to became soluble fibrins that crosslink with each other; the polymers are stabilized by factor VIII and, together with platelets, forms a mass to fill and seal the wound and conclude the clot cascade (Monroe et al., 2010). In addition to its protective role, haemostasis is critical to successful wound healing (Martin, 1997). The blood clot contains fibrin molecules, fibronectin, vitronectin and thrombospondins, forming the provisional matrix as a scaffold structure for the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells and as a reservoir of growth factors (Clark, 1988; Laurens et al., 2006; Monroe et al., 2010; Reinke and Sorg, 2012).

Fibronectin, an extracellular matrix protein, specifically binds a large number of molecules including other components of the extracellular matrix, signalling molecules, and cell adhesion molecules, serving as a chemoattractant for the migration of wound repair cells and as a template for the deposition of collagen fibres (Clark, 1988; Strodtbeck, 2001; Zollinger and Smith, 2017). In addition, platelets influence the infiltration of leukocytes by the release of chemotactic factors. Both platelets and leukocytes release cytokines and growth factors to activate the inflammatory process, stimulate the collagen synthesis, activate the transformation of fibroblasts to myofibroblasts, start the angiogenesis and already support the reepithelialisation process (Barrientos et al., 2008; Reinke and Sorg, 2012; Werner and Grose, 2003).

1.2. Inflammation

The inflammatory phase is an essential phase of healing, characterized by increased vascular permeability, chemotaxis of circulating cells into the wound environment, local release of cytokines and growth factors, and activation of migrating cells (27), which is triggered by a variety of mediators released from injured tissue cells and capillaries, activated platelets and their cytokines, and the by-products of haemostasis (4). Recently, several cell types, which bridge between innate and adaptive immunity, have been shown to play key roles in skin wound healing (28). This phase can roughly be divided into an early phase with predominant neutrophil recruitment and a late phase with predominant migration and maturation of monocytes (14).

In most cases, within a few minutes after injury, neutrophils migrates to the wound tissue, cleanse the wounded area of bacteria and foreign particles and are then extruded with the eschar or phagocytosed by macrophages and monocytes (29, 30). Neutrophis are predominant for the

first few days; however, if the wound becomes infected, neutrophil infiltration continues until infection is crontrolled (4, 28). Both monocytes and skin-resident macrophages are activated by local microenvironmental signals to mature into various subpopulations, defined by their distinct functional phenotypes (31).

Macrophages have many functions including phagocytosis of pathogens and cell debris (32, 33), the promotion and resolution of inflammation, the removal of apoptotic cells and the support of cell proliferation and tissue restoration following injury (34). Besides their immunological functions as antigen-presenting cells and phagocytosis during wound repair, macrophages supposedly play an integral role in a successful healing response through the synthesis and the secretion of growth factors, chemokines and cytokines. Apart from their actual support in wound healing, these molecules keep the healing process intact, as some of them are able to activate the next phase of wound healing (proliferative phase) (13).

Inflammatory phase is essential to hemostasis and recruitment of the innate immune system, which defends us from pathogens and help remove dead tissues (14). However, prolonged inflammation is detrimental and may result in deregulated differentiation and activation of keratinocytes, impeding the progress through the normal stages of wound healing (35). Severe inflammation has also been associated with excessive scarring (36).

1.3. Proliferation and repair

As the inflammation subsides, proliferation becomes predominant to cover the wound surface with new skin (re-epithelialization), restore the vascular network (neovascularization) and repair the tissue structure integrity by filling it with new connective tissue (granulation) (14, 18, 28). Wound (tensile) strength begins to develop during this stage. Key cells for these processes are fibroblasts and keratinocytes (4).

During the proliferation phase, fibroblasts proliferate and migrate into the provisional extracellular matrix of the wound. This is only possible in the presence of angiogenesis, which provides the oxygen and nutrients necessary for fibroblast migration and collagen synthesis. Epithelial cells also proliferate and migrate to close the wound surface (14, 37).

Re-epithelialization of wounds begins within hours after injury and requires migration and proliferation of keratinocytes (28, 30). In a few hours, the existing wound-edge keratinocytes start to migrate. The keratinocytes at the basal layer of the wound-edge and the epithelia stem cells from skin appendages, such as sweat glands and hair follicles, start proliferating (28). Precise stimulus for epidermal cell proliferation and migration is unknown. However, some possibilities exists, such as the absence of neighbour cells at the margin of the

wound (the “free edge” effect), and the local release of growth factors and increased expression of growth-factor receptors (22, 28, 37).

Restoring the network of blood vessels is important, since nutrients and oxygen are needed during wound repair. This process, called angiogenesis, is a complex cascade of cellular, humoral and molecular events in the wound bed to reconnect to the nutritive perfusion (14). Angiogenesis is stimulated by growth factors, tissue hypoxia, and the serine protease thrombin in the wounds, which activate the endothelial cells of existing vessels (4, 28, 38). The activated endothelial cells secrets proteolytic enzymes to dissolve the basal lamina, escape from the existing vessels, proliferate and migrate towards the source of the angiogenic stimulus. These sprouts form vessel lumen, differentiate into arteries and venules and mature by recruitment of pericytes and smooth muscle cells (28, 38).

The last step in the proliferation phase is the development of the acute granulation tissue. At the same time the remodelling phase is already initiated (14). In this stage, the provisional wound matrix formed during haemostasis is replaced by granulation tissue, consisting of a large amount of fibroblasts, granulocytes, macrophages, blood vessels, in complex with collagen bundles, which partially recovers the structure and function of the wounded skin (39). After migrating into the provisional wound matrix, fibroblasts proliferate and produce proteinases (matrix metalloproteinases) to degrade provisional matrix (40, 41). It also deposits collagen and other extracellular matrix components, such as proteoglycans, hyaluronic acid, glycosaminoglycan, and fibronectin, to form granulation tissue (36), which fill up the wound gap and provide a scaffold for cell adhesion, migration, growth and differentiation during wound repair (28, 42, 43).

1.4. Remodelling

Remodelling is the last phase of wound healing and occurs from day 21 to up to 1 year after injury (Reinke and Sorg, 2012). This phase is characterized by the maturation of the granulation tissue into mature connective tissue and/or scar (Strodtbeck, 2001). The wound also develops its final strength during this stage of wound healing (Kerstein, 1997). The key cells for this phase are macrophages and fibroblasts, and the main mechanisms are extracellular matrix reshaping by cross-linking collagens, cell maturation, and program cell death or apoptosis (Clark, 1988; Strodtbeck, 2001).

During the maturation of the wound, the components of the extracellular matrix undergo certain changes. Collagen III, which was produced in the proliferative phase, is now replaced by the stronger collagen I, which is oriented in small parallel bundles and is, therefore, different

from the basket-weave collagen in healthy dermis (Gurtner and Evans, 2000; Reinke and Sorg, 2012). In a normal wound, homeostasis between collagen synthesis and breakdown is achieved within 3 weeks of injury. Clinical appearance of scars can change for up to 2 years post-injury as a result of the maintenance of this equilibrium (Williamson and Harding, 2004).

Mechanical tension and cytokines, for example TGF-β, drive fibroblasts to differentiate into myofibroblasts, which express α-smooth muscle actin and contract the wound (Hinz, 2007), by their multiple attachment to collagen, helping to decrease the surface of the developing scar (Profyris et al., 2012; Tziotzios et al., 2012). Myofibroblasts undergo apoptosis when healing is complete (Xue and Jackson, 2015). Furthermore, the number of new blood vessels and the blood flow decline. A mature avascular and acellular environment is formed (Greenhalgh, 1998), and the acute wound metabolic activity slows down and finally stops (Reinke and Sorg, 2012).

There are certain skin components that will never fully recover after wound closure. Sub epidermal appendages such as hair follicles or sweat glands have no potential to heal or grow back after serious injury. The epidermis of the resultant scar differs from uninjured skin after wound healing due to the lack of rete pegs that are normally anchored into the underlying connective tissue matrix and are responsible for the tight connection of the epidermis to the dermis (Reinke and Sorg, 2012; Robson et al., 2001). Scarring can also be excessive, leading to hypertrophic scars and keloids (Werner and Grose, 2003).

In the first three weeks of the healing process, wounds gain only about 20% of skin final strength (Strodtbeck, 2001). During remodelling, the tensile strength increases from about 20% to a maximum of 70–80% (Williamson and Harding, 2004). This strength increase is due to a much slower rate of accumulation of collagen and, more important, collagen remodelling with the formation of larger collagen bundles and an increase in the number of intermolecular cross-links (Bailey et al., 1975). Nevertheless, wounds never attain the same breaking strength (the tension at which skin breaks) as uninjured skin. At maximal strength, the healed skin can only achieve maximum around 80% of the original tensile strength (Landén et al., 2016; Levenson et al., 1965; Schilling, 1976).

2. Wound Dressings

In 1962, Winter concluded that moisturized wounds in piglets skin epithelizes two times faster than air exposed wounds (Winter, 1962). Since then, much has been learned about wound healing mechanisms and factors that affect them (Cooper, 1990; Cuzzell and Stotts, 1990; Salas Campos et al., 2005; Winter and Scales, 1963), dramatically expanding dressing practices. It is

known that healing is a complex and intricate process, which can be accelerated and enhanced by the use of dressing techniques, products and actives (Cooper, 1990; Kumar et al., 2007).

Thus, wound management is important to avoid those complications, and the use of topical chemotherapy has been fundamental, improving survival of patients with major chronic wounds and burns by decreasing sepsis events. Together with antimicrobial properties, functional and aesthetic effects are also enhanced with drug therapies (Atiyeh et al., 2007; Fraser et al., 2004; Salas Campos et al., 2005). Wound dressings are applied directly on the wound surface (Hajská et al., 2017) and they should ideally: promote removal of excess exudate, maintenance of a moisture environment, protection against contaminants, no trauma or debris left on its removal, pain relief, thermal insulation and no allergic reactions (Abdelrahman and Newton, 2011; Fonder et al., 2008; Seaman, 2002).

Wound dressings have developed over the years from the crude applications of plant herbs, animal fat and honey to tissue engineered scaffolds (Boateng et al., 2008), and more than 3,000 products have been developed to treat different types of wounds by targeting various aspects of healing process (Dhivya et al., 2015). Those different dressing products can be classified in a number of ways depending on, for example, their function, employed material used in the production, dressing physical form, their contact with wound surface, among others (Boateng et al., 2008). They can be also classified as traditional and modern wound dressings (Boateng et al., 2008; Dhivya et al., 2015; Sarabahi, 2012).

Traditional or passive wound dressings were commonly used in the past and though now less widely used, they are still of some benefit in certain clinical settings for wound treatment (Boateng et al., 2008). They are simple products, including topical liquid and semi-solid formulations as well as dry traditional dressings, such as gauze, lint, plasters, bandages (natural or synthetic) and cotton wool (Dhivya et al., 2015; Sarabahi, 2012). They are dry and have no direct effect on the wound except protecting it from contaminations (Dhivya et al., 2015; Sarabahi, 2012).

On the other hand, modern wound dressings have been developed to facilitate wound rather than just to cover it (Dhivya et al., 2015), and their essential characteristic is to retain and create a moist environment around the wound to facilitate wound healing (Boateng et al., 2008). Most modern dressing products are interactive dressings as they interact with the wound bed to provide optimum environment at the wound dressing interface (Sarabahi, 2012). These interactive dressings include semi-permeable film and foam, hydrofibres, hydrogels, hydrocolloids, and alginates (Dhivya et al., 2015; Sarabahi, 2012).

Bioactive or biological dressings are also modern wound dressings and are produced from biomaterials, which play an important role in healing process (Boateng et al., 2008; Dhivya et al., 2015). These dressings are known for their biocompatibility, biodegradability and non-toxic nature (Dhivya et al., 2015), and are derived generally from natural tissues or artificial sources (Bartlett, 1981; Boateng et al., 2008; Dhivya et al., 2015). In some cases, bioactive dressings may be incorporated with active compounds, such as antimicrobials and growth factors for delivery to the wound site, enhancing healing process (Boateng et al., 2008; Dhivya et al., 2015).

3. Synthetic Actives for Wound Healing

Topical antimicrobial therapy sometimes represents an essential part of wound care (Hajská et al., 2017), even though, some studies have shown that dressings in high bactericidal concentration might also delay wound healing due to cytotoxicity (Aziz and Abdul Rasool Hassan, 2017; Hajská et al., 2017; Lineaweaver et al., 1985; Rosanova et al., 2012). The current range of commonly used topical agents include topical antibiotics (bacitracin, mupirocin, neosporin, polymyxin B, nitrofurazone, nystatin), different silver impregnated dressings, iodine solutions, chitosan preparations, acetic acid solution and others (Dai et al., 2010).

3.1. Silver-based

The first topical applications of silver nitrate were probably for the treatment of chronic wounds or ulcers, and dated from the Middle Age (Klasen, 2000). The antimicrobial properties of silver have been recognized for many centuries (Atiyeh et al., 2007; Klasen, 2000). The metal itself is inactive, but when ionized it has broad-spectrum activity against microorganisms (Davies et al., 2017). Due to its antimicrobial activity, silver compounds has been incorporated in wound dressing products, especially on those used on burn wounds (Atiyeh et al., 2007; Castellano et al., 2007; Leaper, 2006).

Silver sulfadiazine is the drug of choice in the infectious period for combating the threat of bacterial infection and preventing wound sepsis (Salas Campos et al., 2005). It has dual antibacterial effects: free silver reacts with both sulfhydryl groups of bacterial enzymes and DNA, and sulfadiazine stops the synthesis of DNA by interrupting the production of folate (Adhya et al., 2014). However, it use has some limitations due to poor aqueous solubility (Dellera et al., 2014) and is *in vitro* cytotoxicity toward fibroblasts and keratinocytes, which can consequently retard wound healing *in vivo* (Rosen et al., 2015). Silver and silver sulfadiazine have also been associated with bacterial resistance development, and some

systemic complications such as neutropenia, methemoglobinemia, renal toxicity, leukopenia, and adverse reactions (Ma et al., 2015; Nasiri and Hosseinimehr, 2017; Shanmugasundaram et al., 2009). Also the efficacy and safety of silver dressings differ and depend on the material used, type of silver compound and its location in the dressing, and total silver content (Davies et al., 2017).

Silver nanoparticles or nanoparticles containing silver have also been studied for wound healing applications. Cotton dress fabrics saturated with silver nanoparticles were tested for 18 days to assess their ability to speed the healing of rats' burn wounds. When compared to fabrics saturated with commercial ointment, the nanoparticles fabrics showed a slightly greater healing efficacy, with higher wound contraction area and better fibril alignments in repaired skin (Pannerselvam et al., 2017).

Chitosan nanoparticles were used as drug carriers for silver sulfadiazine. The results indicate that the formulated dressing presented continuous delivery of SSD that could extend over 24 hours, compared to two hours release offered by the market product. It also presented proven effectivity for Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria and *Candida albicans* on an infected wound (El-Feky et al., 2017).

After conducting a literature review, Davies et al. (2017) find out that scientific and clinical results indicate that clinical, patient-related and economic benefits are associated with the use of silver-containing foam dressings in the treatment of wounds where antimicrobial activity is needed to help manage bioburden. A recent meta-analysis proved that there is evidence base for silver in wound management. Thus, if used selectively and for a limited period, silver not only has antimicrobial effects but is also characterized by an improvement in quality of life and good cost-effectiveness (Dissemond et al., 2017).

3.2. Nitrofurazone

Nitrofurazone is an antibiotic drug, with reported bactericidal activity against a great variety of Gram-positive and Gram-negative bacteria and other microbes which generally cause surface skin infections (Gupta et al., 2013; Zhao et al., 2015), including *Staphylococcus aureus*, *Streptococcus*, *Escherichia coli*, *Clostridium perfringens*, *Enterobacter aerogenes*, and *Proteus organisms* (Murugasu-Oei and Dick, 2000). Due to its bactericidal properties, nitrofurazone have been used as topical formulations, as creams, gels and ointments in the treatment of infected wounds from traumatisms, burns or surgical interventions (De Luca et al., 2010; Vila et al., 2014).

Nitrofurazone have been loaded into hydrogel networks aiming the development of wound dressing (Gupta et al., 2013; Kim et al., 2008; Vila et al., 2014; Zhao et al., 2015). Gupta et al. (2013) synthesized by chemical crosslink technique, a polyvinyl alcohol (PVA)–polyethylene glycol (PEG) semi-interpenetrating hydrogel network (IPN)-based wound dressing system containing nitrofurazone. *In vitro* diffusion studies indicate a relative slow release of drug, attributed to its microencapsulation in the polymeric matrix. PVA-PEG hydrogel with nitrofurazone improve the overall healing rate when tested *in vivo*, as evidenced by histological examinations, significant increase in total protein, hydroxyproline and hexosamine contents, and also a faster wound contraction was observed (Gupta et al., 2013).

PVA hydrogel crosslinked with glutaraldehyde was also impregnated with nitrofurazone for topical application (Vila et al., 2014). Incorporated drug displayed antibacterial activity, being released in a linearly controlled fashion above 6 µg/mL during experiment timeframes of 14 h, a characteristic that may allow its use in occlusive dressings for prolonged periods of time (Vila et al., 2014). Zhao et al. (2015) produced a dual-layer fiber dressings with nitrofurazone by electrospinning, and a controlled drug release profile was also achieved by adjusting nitrofurazone amount in the different layers of the dual-layer dressing. The prepared nanofiber presented satisfactory *in vitro* antibacterial activity against Gram-positive and Gram-negative bacteria and achieved a better performance than commercial non-woven dressing when tested *in vivo* (Zhao et al., 2015).

After evaluate the potential toxic effect of topical antimicrobial agents on cultured murine and human dermal cells, nitrofurazone was considered a semi-toxic agent, while silver sulfadiazine was toxic. This study was designed to test various agents in different application forms, such as solutions, creams, ointments and commercially produced impregnated dressings as well, selecting agents recommended for burn wound care (Hajská et al., 2017).

3.3. Zinc

Zinc is an essential trace element in the human body and its medicinal properties in the form of calamine were documented more than 3,000 years ago (Lansdown et al., 2007). It is an essential component of more than 300 metalloenzymes and over 2000 transcription factors (Gupta et al., 2014), including zinc-dependent matrix metalloproteinases that augment auto-debridement and keratinocyte migration during wound repair (Lansdown et al., 2007). Zinc deficiency of hereditary or dietary cause can lead to pathological changes and has many manifestations ranging from delayed wound healing to immune dysfunction and impairment of multiple sensory systems (Kogan et al., 2017; Lansdown et al., 2007).

Therapeutically, zinc can be used, both topically and in systemic form, for a large number of dermatological disorders (Gupta et al., 2014), once it is known as an important element in wound healing and a common antibacterial agent (Li et al., 2017; Liu et al., 2009). Topical administration of zinc appears to act reducing superinfections and necrotic material via enhanced local defence systems and collagenolytic activity, and the sustained release of zinc ions stimulates epithelialization of wounds in normozincemic individuals (Lansdown et al., 2007). Zinc showed modulating actions on macrophage and neutrophil functions, natural killer cell/phagocytic activity, and various inflammatory cytokines, resulting in anti-inflammatory action (Gupta et al., 2014)

Forty leprosy patients were treated with a phenytoin sodium fine powder zinc oxide paste dressing, that was applied every day. After 4 weeks of daily therapy, 55% patients showed complete clearance of the ulcers, while 82.5% showed development of granulation tissue, and thus phenytoin sodium zinc oxide paste was found to be an efficacious, cost-effective, and well-tolerated alternative therapy (Sehgal et al., 2014). This therapeutically beneficial effect of zinc in chronic cutaneous ulcers may be attributed to its anti-inflammatory and antibacterial properties and its ability to enhance reepithelialization (Gupta et al., 2014).

Li et al. (2017) proposed a novel hydrogel approach by utilizing bioactive ions released from a Zn–Ca–Si ceramic (hardystonite) to crosslink alginate, aiming the development of a bioactive hydrogel with the ability to enhance blood vessel formation and inhibit bacterial growth. Their results demonstrated that the composite hydrogel stimulated proliferation and migration of both fibroblasts and endothelial cells, which are critical for wound healing; and indeed had excellent antibacterial activity, which was attributed to zinc ions. Furthermore, *in vivo* results revealed that the composite hydrogel significantly enhanced wound healing, with histological and immunohistochemical results clearly evidencing the stimulation of blood vessel formation and epithelial formation, which are closely related to the activated cell proliferation and migration (Li et al., 2017).

Zinc oxide nanoparticles are acknowledge antibacterial effect, *in vitro* adhesion between cells and tissues, and pro-angiogenic properties (Barui et al., 2012; Gao et al., 2017; Raghupathi et al., 2011). Thus, they have been applied to different materials and formulations, aiming the development of wound healing dressings (Augustine et al., 2014; Khalid et al., 2017; Kumar et al., 2012; Kumar et al., 2013).

Among these materials, bacterial cellulose have been studied for medical purposes, including its application in wound healing, due to its favorable properties (Jozala et al., 2016), and recently studies aimed to enhance bacterial cellulose characteristics by its impregnation

with bioactive molecules (Khalid et al., 2017; Maneerung et al., 2008). Zinc oxide nanoparticles were successfully impregnated in bacterial cellulose membranes, and exhibited 90%, 87.4%, 94.3% and 90.9% activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Citrobacter freundii*, respectively. In burn mice model, bacterial cellulose-zinc oxide nanocomposites also showed significant healing activity (66%), with fine tissue regeneration proved by histological analyses when compared to bacterial cellulose (Khalid et al., 2017).

Conclusions and Future Perspectives

Wound healing is a well-orchestrated process, comprehending four overlapping and dependent phases, which are regulated by cellular, humoral and molecular mechanisms. This complex and intricate process can be accelerated and enhanced by using dressing techniques, products and actives. Wound management has proven to avoid non-healing complications, and the use of topical chemotherapy has improved survival of patients with major chronic wounds and burns.

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CAPÍTULO II. "NATURAL ACTIVES FOR WOUND HEALING: A REVIEW"

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Abstract

Nature has been a source of medicinal treatments for thousands of years, with the use of plants as prototypes for drug development and for the extraction of active compounds. Skin injuries occur regularly in everyday life, and the human skin has the ability to promote repair spontaneously under healthy conditions. However, some intrinsic and external factors may interfere with skins' natural ability, leading to non-healing lesions and chronic wounds, which directly affect health and quality of life. Thus, attention should be given to this health problem, using an appropriated management when necessary. In this scenario, phytotherapy may be an option for cutaneous wound treatment, although further high quality studies are needed to firmly establish the clinical efficacy of plants. This article reviews traditionally used natural actives for wound healing, highlighting their characteristics and mode of action.

Keywords: wound healing; natural actives; topical actives

Introduction

The largest organ of the human body is the skin, accounting for approximately 15% of total body weight in adults (Mulholland et al., 2017; Tobin, 2006). It acts as a protective barrier against the external environment and helps to prevent dehydration (Mulholland et al., 2017), which highlight the importance to maintain its integrity. Reconstructing functional skin after a wound remains a challenge due to the complexity of healing, involving orchestrated cell-signalling events and biochemical cascades (Berthet et al., 2017).

After damage, human skin promotes spontaneous repair by a four-stage process that results in the formation of non-functional fibrotic tissue (Gurtner et al., 2008). Impairments at any one or more of these stages can lead to compromised healing (Mulholland et al., 2017), which can lead the wound to enter in a chronic state, affecting patients' health and quality of life (Pereira and Bártolo, 2016; Abdelrahman and Newton, 2011). Thus, wound management is important to avoid complications, comprising appropriated topical pharmacotherapy and dressings (Fraser et al., 2004; Atiyeh et al., 2007; Salas Campos et al., 2005), which make chronic wounds a major target for medical technological development (Gueldner et al., 2017). Conventional therapies include the use of synthetic antibiotics and healing promoters (Hajská et al., 2017), and dressings that are used to protect dermal and epidermal tissues (Felgueiras and Amorim, 2017). However, these treatments and dressing are mostly inadequate for chronic wounds (Mulholland et al., 2017).

For thousands of years, nature has been a source of medicinal treatments, and plant-based systems continue to play an essential role in primary healthcare around the world, with natural compounds been used in skin wound care particularly due to their anti-inflammatory, antimicrobial, and cell-stimulating properties (Pereira and Bártolo, 2016; Pazyar et al., 2014). Many plants and their extracts have been traditionally used due to their great potential for wounds management and treatment, with these agents inducing healing and tissue regeneration through multiple connected mechanisms (Maver et al., 2015). In addition, natural agents may be useful for handling of abnormal healing, such as keloids and hypertrophic scars (Pazyar et al., 2014).

Phytotherapy may open new avenues for therapeutic intervention on cutaneous wounds (Pazyar et al., 2014). However, information concerning quantitative human health benefits of plant-based medicines is still rare or dispersed, limiting their proper evaluation, with further high quality studies needed to firmly establish the clinical efficacy of plants. In this scenario, more recently, researches have been conducted to prove efficacy of herbal medicines and to better understand their action (Maver et al., 2015; Pazyar et al., 2014; Das et al., 2016; Das et

al., 2017). Based on the exposed above and the plant-based systems trend for wound management, here we reviewed the characteristics and mode of action of natural actives, using Web of Knowledge and PubMed databases to search articles from the last 10 years, choosing actives based on their traditional and topical use. In this review, articles were chronologically cited; however, for the sake of clarity they were classified as experimental (*in vitro* and *in vivo*) and clinical on Table II.1. In addition, an overview of the selected actives is presented on Table II.2.

Table II.1. Reviewed articles classification according with study type.

Plant/Natural Active	Experimental *in vitro*	Experimental *in vivo*	Clinical
Bromelain	(Dutta and Bhattacharyya, 2013; Ali et al., 2015; Praveen et al., 2014; Manosroi et al., 2014; Ataide et al., 2017b; Ekambaram et al., 2017)	(Singer et al., 2010; Wu et al., 2012; Hu et al., 2011; Dutta and Bhattacharyya, 2013)	(Krieger et al., 2012; Cordts et al., 2016; Rosenberg et al., 2014; Rosenberg et al., 2004; Koller et al., 2008; Schulz et al., 2017; Blonstein, 1960)
Propolis	(Boufadi et al., 2014; Falcão et al., 2014; Al-Waili et al., 2015)	(Iyyam Pillai et al., 2010; Hozzein et al., 2015; Al-Waili et al., 2015; Corrêa et al., 2017; McLennan et al., 2008)	(Henshaw et al., 2014)
Centella asiatica	(Hong et al., 2005; Shukla et al., 1999b; Gohil et al., 2010; Incandela et al., 2001; Zhang et al., 2016)	(Shukla et al., 1999b; Sawatdee et al., 2016; Gohil et al., 2010; Gul Satar et al., 2013; Sunilkumar et al., 1998; Rosen et al., 1967; Incandela et al., 2001; Poizot and Dumez, 1978; Shukla et al., 1999a; Suguna et al., 1996; Zhang et al., 2016)	(Gohil et al., 2010; Saeidinia et al., 2017)
Punica granatum L.	(Tanveer et al., 2015)	(Murthy et al., 2004; Hayouni et al., 2011; Yan et al., 2013; Pirbalouti et al., 2010; Mo et al., 2014; Nasiri and Hosseinimehr, 2017)	(Fleck et al., 2016)
Rosmarinus officinalis L.	(Pérez-Sánchez et al., 2014; Liakos et al., 2017; Liakos et al., 2014; Sienkiewicz et al., 2017; Tawab et al., 2015)	(Abu-Al-Basal, 2010; El-Mehi and El-Sherif, 2015; Amaral et al., 2013; Benincá et al., 2011)	
Calendula officinalis	(Dinda et al., 2016; Dinda et al., 2015; Nicolaus et al., 2017; Szakiel et al., 2008; FIT et al., 2009; Hussain et al., 2012)	(Zitterl-Eglseer et al., 1997; Parente et al., 2012; Patrick et al., 1996; Fronza et al., 2009; Dinda et al., 2016)	(Leach, 2008; Buzzi et al., 2016; Cioinac, 2016)

Table II.2. Overview of main information about reviewed plant/natural actives.

Plant/Natural Active	Origin	Characteristics	Pharmaceutical Form	Reference
Bromelain	Central and South America	Proteolytic and non-enzymatic substances found mainly in pineapple (*Ananas comosus*)	Emulsions, hydrogels, biocompatible polymer membranes	(Krieger et al., 2012; Wu et al., 2012; Singer et al., 2010; Ekambaram et al., 2017)
Propolis	World	Resinous substance rich in polyphenols	Liquid, ointment	(Iyyam Pillai et al., 2010; Hozzein et al., 2015; Henshaw et al., 2014; Corrêa et al., 2017)
Centella asiatica	Tropical and subtropical countries	Saponins and their sugar esters (asiaticoside)	Liquid, ointment, porous microparticles	(Saeidinia et al., 2017)
Punica granatum L.	Eastern Mediterranean and Middle East	Polyphenols, specially tannins	Gel, ointment	(Murthy et al., 2004; Hayouni et al., 2011; Yan et al., 2013; Nasiri and Hosseinimehr, 2017; Fleck et al., 2016)
Rosmarinus officinalis L.	Mediterranean and Asia	Phenolic diterpenes, caffeoyl derivates and flavones	Liquid, impregnated dressings, micro and nanofiber membranes	(Pérez-Sánchez et al., 2014; Abu-Al-Basal, 2010; El-Mehi and El-Sherif, 2015)
Calendula officinalis	Southern Europe	Triterpenoids, saponins and flavonoids	Liquid, creams, ointments	(Fronza et al., 2009; Parente et al., 2012; Dinda et al., 2016; Nicolaus et al., 2017; Patrick et al., 1996; Buzzi et al., 2016; Cioinac, 2016)

1. Bromelain

Pineapple has been used as part of traditional folk medicine since ancient times (Rathnavelu et al., 2016), and bromelain has been chemically known since 1875 and used as a

phytomedical compound ever since (Taussig and Batkin, 1988; Lourenço et al., 2016). The term "bromelain" is used to describe a mixture of proteolytic enzymes and non-enzymatic substances found in Bromeliaceae species, mainly in pineapple (*Ananas comosus* L.) and its stem, fruit and leaves (Spir et al., 2015; Maurer, 2001; Rathnavelu et al., 2016). However the complete molecular mechanism of action of bromelain has not been completely identified, bromelain gained universal acceptability as a phytotherapeutic agent, being widely administered for its well-recognized properties, such as its anti-inflammatory, antithrombotic and fibrinolytic affects, anticancer activity and immunomodulatory effects, in addition to being a wound healing and circulatory improvement agent (Rathnavelu et al., 2016; Ataide et al., 2017a; Spir et al., 2015; Lourenço et al., 2016; Taussig and Batkin, 1988; de Lencastre Novaes et al., 2016; Maurer, 2001). In Europe, bromelain is approved for oral and topical use, mainly for surgical wounds, inflammation due to trauma and surgery, and debridement of deep burns (Muhammad and Ahmad, 2017).

Various studies refer to bromelain debriding action, removing the necrotic layer of epidermis, which could serve as an excellent culture medium for opportunistic pathogens (Maurer, 2001; Ekambaram et al., 2017; Houck et al., 1983; Singer et al., 2010; Wu et al., 2012; Sheridan et al., 1994; Hu et al., 2011; Cordts et al., 2016; Rosenberg et al., 2014; Rosenberg et al., 2004). Local application of bromelain has been shown to be rapid, effective, non-invasive, safe, easily performed at the bedside with minimal or no blood loss and negligibly interfere with natural wound healing processes (Muhammad and Ahmad, 2017; Koller et al., 2008). Bromelain also accelerates the healing of burn wounds in humans (Krieger et al., 2012), showing antioxidant and antimicrobial activities (Dutta and Bhattacharyya, 2013; Ali et al., 2015; Praveen et al., 2014; Manosroi et al., 2014; Ataide et al., 2017b).

Commercial enzymatic debriding agents are available and some previous studies reported their efficacy mainly in the treatment of burn wounds (Cordts et al., 2016; Rosenberg et al., 2014; Schulz et al., 2017), and to clear bruises and hematomas on boxers (Blonstein, 1960). Even though, other attempts have been made to develop new topical formulations and wound dressings containing bromelain.

Oil-in-water emulsions were developed to vehicle extracted and commercial available bromelain, however its enzymatic activity decrease during time and depending on storage conditions (Lourenço et al., 2016; Spir et al., 2015). Hydrogel dressings with bromelain were also developed, using synthetic and natural polymers. Poly(N-isopropylacrylamide)-co-acrylamide hydrogels have been developed and presented an ability of loading 56% of bromelain from a bromelain solution and releasing up to 91% of the retained bromelain. Ataide

et al. (2017a) described a hydrogel formulation, using alginate and Arabic gum, for bromelain loading and release. The resulted hydrogel absorbed 19% of bromelain from a bromelain solution after 4 hours of contact at 4°C, and enzyme immobilization ocurred by hydrogen bond formation.

Ekambaram et al. (2017) describes a combinational multiphasic delivery system produced by coaxial electrospinning of different biocompatible polymers, with optimization of the ratio and specificity of polymers for specific bio-function, using bromelain as a debriding agent, and salvianolic acid B as an angiogenesis and re-epithelialization stimulator. The *in vitro* release profile illustrated the sustained release of debriding protease and bioactive component in a timely fashion. The fabricated scaffold showed angiogenic potential through in vitro migration of endothelial cells and increased new capillaries from the existing blood vessel in response to an *in ovo* chicken chorioallantoic membrane assay. With the designed fibre also achieved an accelerated *in vivo* wound healing on the full thickness rat skin wound model (Ekambaram et al., 2017).

2. Propolis

Propolis is a natural product found in plant materials and is processed by worker bees by mixing the material with salivary enzymes and wax (Chan et al., 2013), resulting in a polyphenol-rich resinous substance, which has the purpose of protecting beehives against insects and infectious agents (Boufadi et al., 2014; Ghisalberti, 1979). Propolis is available as a dietary supplement, in products for the protection of health and prevention of diseases, in biopharmaceuticals, and as a constituent of (bio)cosmetics (de Groot, 2013). Due to polyphenol-rich profile, propolis has well-established properties, such as anti-inflammatory, antioxidant (Boufadi et al., 2014) and antibacterial (Falcão et al., 2014), which make it an attractive candidate for wound healing (Hozzein et al., 2015).

The study of Iyyam Pillai et al. (2010) evaluates wound healing potential of Indian propolis on excision wounds induced in experimental rats. Propolis ointment topical application improved the wound contraction when compared to the control group. The determination of hydroxyproline, hexosamine, uronic acid, DNA, RNA and protein levels in the wound matrix revealed the pro-healing effects of propolis, showing results comparable with nitrofurazone. The observed prophylactic action of propolis in wound healing may be due to the presence of biologically active ingredients such as flavonoids, phenolic acids, terpenes, benzoic acids, amino acids and vitamins (Iyyam Pillai et al., 2010).

Hozzein et al. (2015) investigated the effects of propolis topical application on the healing and closure of diabetic wounds in a streptozotocin-induced type I diabetic mouse model. Interestingly, compared with untreated diabetic mice, propolis topical application enhanced the closure of diabetic wounds and the production of collagen via the TGF-β1/Smad2,3 signalling axis in wounded tissues, and also decreased the levels of IL-1β, IL-6, TNF-α and MMP9 to near normal levels. Their findings help to reveal the molecular mechanisms underlying the improved healing and closure of diabetic wounds following topical propolis application.

A review from 2015 summarized previous and recently published papers of the effects of two bee products, propolis and bee venom, on the wound healing, using mainly results obtained from preclinical experimentation. These data indicated that bee venom and propolis have potential therapeutic properties in chronic diabetic wound, and the use of propolis and/or bee venom can be new effective intervention in clinical practice. However, their therapeutic effects should be proved by clinical studies, focusing on the advantage of using these agents over the current interventions, particularly in respect of therapeutic activities, safety, and cost effectiveness (Al-Waili et al., 2015).

Corrêa et al. (2017) studied the effect of red propolis oral administration on inflammation and wound healing in mice, using a tissue repair model. After propolis administration it was observed that treated group presented faster wound closure, with less neutrophils and macrophages in tissue analysis, and reduced inflammatory cytokine production (TGF-β, TNF-α, and IL-6). Their findings suggest a positive role of red propolis oral administration in suppressing the inflammatory response during tissue repair, acting in the wound healing process (Corrêa et al., 2017).

In addition, a pilot study on human diabetic foot ulcer healing was conducted to determine propolis efficacy and tolerance. A previous study from the same group had showed that a single application of topical propolis normalized ulcer closure rate and reduced persistent neutrophil infiltration and elastase activity in a preclinical study, using diabetic rodent model of full thickness cutaneous wound healing (McLennan et al., 2008). During clinical study, serial consenting subjects had topical propolis applied at each clinic review for 6 weeks. Results showed that ulcer area was reduced by a mean 41% in the propolis group compared with 16% in the control group at week 1, and by 63 vs. 44% at week 3, respectively. In addition, 10 vs. 2% of propolis treated vs. control ulcers had fully healed by week 3. Post-debridement wound fluid active MMP-9 was significantly reduced, as were bacterial counts, and no adverse effects from propolis were reported (Henshaw et al., 2014).

3. *Centella asiatica*

Centella asiatica is used as a traditional herbal medicine in Asiatic countries for hundreds of years to improve wound healing, and it is becoming popular in the West (Brinkhaus et al., 2000; Hong et al., 2005). It is found in most tropical and subtropical countries growing in swampy areas, including parts of India, Pakistan, Sri Lanka, Madagascar, and South Africa and South pacific and Eastern Europe (Gohil et al., 2010).

The primary active constituents of *Centella asiatica* are saponins (also called triterpenoids) and their sugar esters, which include asiaticoside, Asiatic acid, and madecassic (Gohil et al., 2010; Brinkhaus et al., 2000). These components are pre-clinically effective on systemic scleroderma, abnormal scar formation, and keloids (Hong et al., 2005). Asiaticoside is the main active ingredient of *Centella asiatica* and exhibits significant wound-healing activity in normal and delayed-healing models (Shukla et al., 1999b).

Several studies demonstrated the effect of *Centella asiatica* in wound healing when topically applied (Sawatdee et al., 2016; Gohil et al., 2010; Gul Satar et al., 2013; Hong et al., 2005). Its mechanism of action has been attributed to increase cellular proliferation and collagen synthesis at the wound site (Sunilkumar et al., 1998; Pazyar et al., 2014), increase in angiogenesis (Rosen et al., 1967; Incandela et al., 2001), and it has an effect on keratinization, which aids in thickening skin in areas of infection (Poizot and Dumez, 1978).

Asiaticoside enhances the induction of antioxidants enzymes at an initial stage of wound healing (Shukla et al., 1999a), an increase in hydroxyproline, in tensile strength, and in collagen content, and better epithelization (Shukla et al., 1999b). The extract increased cellular proliferation and collagen synthesis at the wound site, as evidenced by increase in DNA, protein and collagen content of granulation tissues, it were also found to epithelialize faster with higher rate of wound contraction (Suguna et al., 1996).

A randomized controlled comparison between silver sulfadiazine and *Centella asiatica* ointments was carried out in partial thickness burning patients. Burn wounds were treated once daily at home and the wounds were evaluated until complete healing occurred and at the admission. All objective and subjective signs, mean of re-epithelialization and complete healing were significantly better in the group treated with *Centella* rather than sulfadiazine group ($p<0.05$). Also, there was no infection in *Centella* group (Saeidinia et al., 2017).

However, the low solubility and oil-water partition coefficient of asiaticoside lead to reduced effect and limited application (Zhang et al., 2016). Asiaticoside is a pentacyclic triterpene with big molecular weight of 959.12 g/mol and it is difficult to be ionized (Zhang et al., 2011). Porous microsphere was shown a novel carrier for the sustained delivery of poorly

soluble asiaticoside, with absorption and therapeutic effects improved. Asiaticoside-microsphere is a promising topical preparation with excellent regenerative effects for the wound therapy (Zhang et al., 2016).

4. *Punica granatum* L.

Punica granatum, commonly recognized as pomegranate, belongs to the Punicaceae family and is considered as a "healing food" from old times because of its useful effects in curing various diseases (Rahimi et al., 2012; Tanveer et al., 2015; Hayouni et al., 2011; Arun and Singh, 2012). In fact, the pomegranate was widely used in folk medicine for the eradication of parasites, as a vermifuge and anthelminthic, useful in dysentery and ulcer (Lansky and Newman, 2007). It is also used for the treatment of aphthae, diarrhoea, acidosis, haemorrhage, respiratory pathologies, hepatic damage, snakebite, and as an antipyretic, reducing fever (Hayouni et al., 2011; Tanveer et al., 2015). Further, its antibacterial, anticonvulsant, anti-inflammatory, antifungal, immunomodulatory, cardio-protective, antimutagenic, antispasmodic and antidiabetic activities have also been documented (Singh et al., 2009).

Polyphenols, especially tannins, are the major components of pomegranate responsible for the healing activity (Arun and Singh, 2012; Tanveer et al., 2015). Pomegranate methanolic extract was incorporated into a gel formulation in different concentrations, and applied in excision wound on the skin of Wistar rats. The group treated with 5.0% gel showed good healing compared with negative and positive controls, with a twofold increase in the hydroxyproline amount. Also healing time in treated animals were smaller than in animals receiving blank gel (10 days in the first group against 16-18 in the second) (Murthy et al., 2004).

An ointment containing 5% (w/w) pomegranate peel methanolic extract was also formulated and applied once a day for 10 consecutive days in wounded guinea pigs models. The ointment significantly enhanced the wound contraction and the period of epithelialization as assessed by the mechanical (contraction rate, tensile strength), the biochemical (increasing of collagen, DNA and proteins synthesis) and the histopathological characteristics. In addition, the extract showed antioxidant activity as strong as natural and synthetic compounds, and showed significant antibacterial and antifungal activity against almost various bacteria (Hayouni et al., 2011).

Yan et al. (2013) showed the ability of pomegranate peel polyphenols gel to increase fibroblast infiltration, collagen regeneration, vascularization, and epithelialization in the wound area of alloxan-induced diabetic rats. It also showed that pomegranate gel-treated diabetic rats showed increased contents of hydroxyproline, production of nitric oxide, and activities of nitric

oxide synthase and increased expressions of TGF-β1 (transforming growth factor-β1), VEGF (vascular endothelial growth factor), and EGF (epidermal growth factor) in wound tissues (Yan et al., 2013).

Previously, Pirbalouti et al. (2010) had compared the efficacy of treatment with *Punica granatum* and *Malva sylvestris* diethyl ether extracts with nitrofurazone (standard drug) in alloxan-induced diabetic rats, based on wound area relative and histopathological characteristics. The results of study showed that the extract ointments of *M. sylvestris* and *Punica granatum* effectively stimulate wound contraction as compared to control group and other groups (Pirbalouti et al., 2010). Increase in tensile strength of incision wound, accelerated wound contraction of excision wound and burn wound, enhanced synthesis of collagen and inhibited neutrophil infiltration was also observed in a study comparing pomegranate peel extract with its major antioxidant constituent, ellagic acid, in three rat dermal wound models (Mo et al., 2014).

The efficacy of *P. granatum* flower extract was compared to silver sulfadiazine for treating thermal burn injuries in rats. The decrease in the average size of wounds on day 15 was higher in rats treated with creams containing pomegranate extract than in rats treated with cream containing sulfadiazine. In addition, the wounds completely healed on day 25 in rats treated with creams containing *P. granatum* flower extract compared with other groups, indicating that pomegranate flower extract promoted wound healing in rats and could be used for managing burn injuries (Nasiri and Hosseinimehr, 2017).

A case study analysing the effect produced by an officinal formulation of ethanolic extracts of *Punica granatum* peels on a non-healing chronic ulcer, with complete closure of the chronic ulcer, has been reported. A 76-year-old woman presented with the chief complaint of a non-healing recurrent ulcer on the left leg with an area measuring 23.52 cm^2, with pain and swelling. The ulcer had not responded to consistent conventional treatment for more than one. The angiologist prescribed a officinal formulation of a 2% (w/w) *Punica granatum* peel ethanolic extract based on a hydrophilic cream and zinc oxide, to be applied once a day. Within six weeks, the ulcer had decreased to one quarter of its original size and had completely healed six weeks later, needing 90 applications for complete healing. No adverse effects of the formulation were observed (Fleck et al., 2016).

5. *Rosmarinus officinalis* L.

Rosmarinus officinalis L., popularly known as rosemary (Lamiaceae), is an evergreen shrub with aromatic needle-like leaves native to the Mediterranean and Asia. Currently it is

cultivated in temperate locations around the world as a decorative garden plant and culinary herb (Satyal et al., 2017). There are several listed varieties of *R. officinalis*, and numerous cultivars have been developed as well, which leads to a variety of chemical compositions. Rosemary extracts contain a number of phytochemicals, including phenolic diterpenes (carnosic acid, carnosol, and 12-O-methylcarnosic acid), caffeoyl derivatives (rosmarinic acid), and flavones (isoscutellarein 7-O-glucoside and genkwanin) (del Baño et al., 2003). While rosemary essential oils can contain α-pinene, 1,8-cineole, verbenone, borneol, camphor, and limonene (Satyal et al., 2017). The biological activities of *R. officinalis* essential oils doubtless depend on its chemical compositions.

Pazyar et al. (2014) point rosemary as one of the common herbs used in cutaneous wound healing, and its aqueous extract and essential oil efficacy in healing was assessed in alloxan-induced diabetic BALB/c mice. Significant differences ($p < 0.01$) between treated and control groups were observed at different aspects of diabetic wound healing process, such as a reduction in inflammation and an enhancement in wound contraction, re-epithelialization, regeneration of granulation tissue, angiogenesis and collagen deposition in the treated wounds. However, essential oil from *Rosmarinus officinalis* exhibited superior significant healing effect over the aqueous extract, when topically applied on the wound of diabetic mice (Abu-Al-Basal, 2010).

Rosemary showed a protective effect against acrylamide-induced gastric toxicity via reducing oxidative stress, apoptosis and inflammation as well as accelerating the healing process (El-Mehi and El-Sherif, 2015). Acrylamide toxicity has been extensively studied, and Mohamed Sadek (2012) has shown that acrylamide significantly increased lipid peroxidation and decreased antioxidative defence systems in gastric mucosa. Rosemary administration increased antioxidant marker enzymes and decreased lipid peroxidation in acrylamide-treated gastric mucosa. These reduced oxidative stress was accompanied with down-regulation of caspase-3 and inducible nitric oxide synthase expression (El-Mehi and El-Sherif, 2015). The anti-inflammatory effect of rosemary extract was also confirmed by microscopic evidence of decreased inflammatory cell infiltrate in relation to samples from rats treated with acrylamide alone (El-Mehi and El-Sherif, 2015; Amaral et al., 2013; Benincá et al., 2011).

Due to its antioxidant activity, rosemary extract was also investigate for its protection against UV harmful effects on human keratinocytes *in vitro*, alone and in combination with citrus extract. Rosemary and citrus extracts have individually demonstrated cellular protective properties against the damaging radical species induced by UVB radiation, however keratinocytes survival after UVB radiation was higher in treatments using the combination of

extracts, indicating potential synergic effects. The combination of extracts also decreased UVB-induced intracellular radical oxygen species and prevented DNA damage in human keratinocytes by comet assay (Pérez-Sánchez et al., 2014).

Besides its antioxidant activity, antibacterial, antifungal, and anti-inflammatory properties have been reported for plant-derived essential oils, including rosemary essential oil, which made those oils attractive for topical application (Liakos et al., 2017; Raut and Karuppayil, 2014; Liakos et al., 2014; Lang and Buchbauer, 2012; Sienkiewicz et al., 2017). Tawab et al. (2015) demonstrate a synergic interaction between rosemary with antimicrobial agents against *S. aureus in vitro*. Thus, rosemary oil have been applied in wound dressings (Liakos et al., 2017; Liakos et al., 2014; Casanova et al., 2016).

Some properties of rosemary extracts, such as antioxidant, anti-carcinogenic and anti-inflammatory, have been attributed to rosmarinic acid. However, its instability, poor solubility in water and low partition coefficient constrain its transport across biological barriers, the inclusion in a cosmetic formulation and the efficacy of the antioxidant, and thus chitosan and modified chitosan microparticles were developed by spray-drying, aiming to overcome those issues (Casanova et al., 2016). Spherical microparticles, with a regular shape and an average diameter of 4.2 μm and 7.7 μm were obtained, using chitosan and modified chitosan as encapsulating agent, respectively. Modified chitosan microparticles presented a slower release of rosmarinic acid in oil than in aqueous medium, while chitosan microparticles showed a faster release in both mediums (Casanova et al., 2016).

Rosemary and oregano essential oils properties were combined with the cellulose acetate nanofibers properties, aiming the development of a wound dressing with antimicrobial activity using the electrospinning technique (Liakos et al., 2017). Electrospun produced micro and nanofibers with approximately 700–1500 nm in diameter. Both essential oils were efficiently incorporated into the electrospun fibres, which were uniform, continuous and free of any defects. Fibres retained antibacterial activity of rosemary and oregano oils and this activity was dose-dependent. The activity also depend on the used microorganism, being higher for *C. albicans* and *E. coli* than for *S. aureus* (Liakos et al., 2017).

6. *Calendula officinalis*

Calendula officinalis, also know as marigold, is a common garden plant with large yellow and orange flowers, native to Southern-Europe (Leach, 2008). For centuries, *Calendula* flowers have been used to treat a number of clinical conditions, specifically, the treatment of dermatological disorders, either in the form of infusions, tinctures, liquid extracts, creams or

ointments, or in one of a number of skin and hair products available over-the-counter across the globe (Final Report 2001; Leach, 2008).

Marigold clinical effects are attributed to a number of chemical components, mainly the triterpenoids (Leach, 2008). Among this triterpenoids, faradiol and its esters presented dose-dependent anti-oedematous activity. Furthermore, faradiol showed the same effect as an equimolar dose of indomethacin (Zitterl-Eglseer et al., 1997). Some other constituents identified in *Calendula* such as the saponins, micronutrients, flavonoids, and polysaccharides, can also be responsible for the anti-oedematous, anti-inflammatory, antioxidant, and wound healing effect of the plant (Leach, 2008; Ahmed et al., 2003).

After a systematic review of the literature to evaluate clinical effectiveness of *Calendula*, Leach (2008) concluded that marigold presents properties that are related to wound healing. Clinical data support several of these properties using topical calendula applications on inflammation, microbial load and epithelization. However, the available data was considered weak and author pointed the need of further investigation using more rigorous clinical trials (Leach, 2008). Another literature review pointed the use of *C. officinalis* in wound healing trough neovascularization and epithelization mechanisms (Pazyar et al., 2014).

C. officinalis presented angiogenic properties, when evaluated through the chorioallantoic membrane and cutaneous wounds in rat models, using macroscopic, morphometric, histopathologic, and immunohistochemical analysis (Parente et al., 2012). The same property was previously demonstrated using stereomicroscopy (Patrick et al., 1996), where the number of micro vessels in *calendula*-treated membranes were statistically significantly higher than in the control group. Also in this study, the histological sections of chorioallantoic membranes were also examined for the presence of hyaluronan, a tissue glycosaminoglycan associated with neovascularization, and all calendula treated membranes were positive for hyaluronan, different from the control group (Patrick et al., 1996).

Marigold extracts stimulate proliferation and migration of 3T3 fibroblasts in a scratch assay (Fronza et al., 2009). In this study, hexane and ethanolic extracts from *Calendula officinalis* as well as the triterpenoids faradiol myristate and palmitate were applied in monolayers of Swiss 3T3 albino mouse fibroblasts, using platelet-derived growth factor as positive control, and antimitotic mitomycin C to differentiate between proliferation and migration. Both extracts stimulated proliferation and migration of fibroblasts at low concentrations (10 μg/mL). Faradiol myristate and palmitate gave comparable stimulation rates at an almost 50 μg/mL concentration, indicating that they contribute partially, but not most significantly to the wound healing effects of calendula preparations (Fronza et al., 2009).

Dinda et al. (2016) studied hydroethanolic extract and its active fraction (water fraction of hydroethanol extract) on primary human dermal fibroblasts *in vitro* and on excisional wounds of BALB/c mice. Their results showed that both samples significantly stimulated the proliferation and the migration of fibroblasts *in vitro*, up regulating the expression of connective tissue growth factor and α-smooth muscle actin. In vivo, treated mice groups showed faster wound healing and increased expression of the same proteins present during granulation (Dinda et al., 2016). *C. officinalis* tincture also stimulated both proliferation and migration of fibroblasts in a statistically significant manner in a PI3K-dependent pathway (Dinda et al., 2015).

Nicolaus et al. (2017) analysed the molecular mechanism of the wound healing effects of *Calendula* extracts through *in vitro* studies. Their results showed that in the *in vitro* model used, the *Calendula* flowers n-hexanic and the ethanolic extracts influence the inflammatory phase, activating the transcription factor NF-κB and increasing the amount of the chemokine IL-8 in human immortalized keratinocytes. The migration of the keratinocytes during the new tissue formation phase was only marginally influenced by the extracts presence in the scratch assay. However, the ethanolic extract inhibited the activity of collagenase *in vitro* and enhanced the amount of collagen in the supernatant of human dermal fibroblasts, indicating that the granulation tissue was affected. Those findings suggest that marigold may have an impact on the inflammatory phase and the new tissue formation phase (Nicolaus et al., 2017).

Even though, the active fraction and/or compounds of *C. officinalis* responsible for wound healing are not known yet (Nicolaus et al., 2017; Dinda et al., 2016), some studies suggest that this activity may be due to the presence of flavonoids, such as rutin and quercetin, along with other small molecules, as amino acids and polyphenols (Dinda et al., 2016; Zbuchea et al., 2016). Phytochemical studies indicated that the roots, stem and leaves of *C. officinalis* contain secondary metabolites such as alkaloids, flavonoids, saponins, anthraquinone, terpenoids, tannins, and others (Hussain et al., 2012; Arora et al., 2013).

Clinical reports have used *C. officinalis* extract and ointment on patients with non-healing venous leg ulcer (Buzzi et al., 2016) and patients diagnosed with diabetes and various injuries (micro traumas) on their lower and upper limbs (Cioinac, 2016), respectively. In both cases, the results were positive, with complete reepithelization; infection progress blocked; reducing itching, redness, pain, dryness, and healing time; disappearance of various scars; regrowth of hair on the legs; and no adverse events were observed (Cioinac, 2016; Buzzi et al., 2016).

Antibacterial activity of marigold have also been reported. Oleanolic acid isolated from marigold inhibited bacterial growth and survival, influenced cell morphology and enhanced the autolysis of Gram-positive bacteria (*B. megaterium*, *L. monocytogenes* and *S. epidermidis*) suggesting that bacterial envelopes are the target of its activity (Szakiel et al., 2008). *C. officinalis* essential oil presented an inhibition diameter area of 21.35 mm on *S. aureus* strains isolated from animal lesions, when using 30µL concentration. However, this inhibition diameter decrease to 0.6 mm when using 3µL concentration (FIT et al., 2009). However, a different behaviour was observed by Chaleshtori et al. (2016), where *C. officinalis* harboured the highest antibiotic effects on the Gram-negative bacteria, presenting an inhibition diameter of 13.31 and 10.22 mm for *E. coli* and *P. aeruginosa*, respectively; and 3.14 mm for *S. aureus*.

Hussain et al. (2012) studied the antibacterial and anti-fungal activities of the methanol and its sub fractions of chloroform, ethyl acetate, and water extracts of the roots, stem and leaves of *Calendula officinalis*. Promising results against both the Gram-positive and Gram-negative bacterial were shown, being comparable with the standard antimicrobiotic gentamicin (10 µg/disc). The methanolic extracts showed inhibitory effects of 16 mm each of the stem and leaves samples against *S. typhymurium* and *S. aureus*. The water extract of leaves has 17 mm against *S. typhymurium* and very high activity among all the extracts 18 mm inhibition of the roots against *E. coli*. The antifungal activity of the extracts showed significantly variable results against the tested *Aspergilla fumigates*, *Fusarium solani*, *Aspergillus niger*, and *Aspergilla flavus* (Hussain et al., 2012).

Conclusion and Future Trends

Skin, the largest human organ, acts as the main barrier against external environment, protecting the body from dehydration and infections (Mulholland et al., 2017). Precisely because of its barrier function, skin is daily exposed to factors that can injury it, resulting in acute and even chronical wounds. Wound healing is a natural and well-orchestrated process that can be compromised under specific conditions (Guo and DiPietro, 2010; Groeber et al., 2011).

Many authors have presented the successful use of natural compounds on inducing cell lines proliferation and migration *in vitro* and good re-epithelization using *in vivo* models. Bromelain, propolis, *Centella asiatica*, *Punica granatum* L., *Rosmarinus officinalis* L., and *Calendula officinalis* L. were selected since they are present in several different products in Brazil. Moreover, those actives were well known in folk culture, stimulating use. Nevertheless these are not the only actives being researched, and there are recent papers on *Polygonum*

aviculare L. (Seo et al., 2016), *Hancornia speciose* (Geller et al., 2015), *Caesalpinia sappan* (Tewtrakul et al., 2015), *Morus alba* (Kim et al., 2015), and Copaiba oil (Wagner et al., 2017), for example.

A lot of effort has been done to demonstrate the potential application of natural extracts and their isolated actives for the development of formulations aiming their application in wound management. Although it is still needed further high quality studies to firmly establish the clinical efficacy of plants.

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Competing Financial Interests

The authors declare no competing financial interests.

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CAPÍTULO III."BROMELAIN-LOADED NANOPARTICLES: A COMPREHENSIVE REVIEW OF THE STATE OF THE ART"

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Abstract

Stem bromelain is a common available cysteine protease derived from pineapple (*Ananas comosus* L.). Bromelain finds widespread applications in several areas, such as medicine, health, food, and cosmetics, and its strong proteolytic activity supports its future application in many additional fields. However, most proteins and/or enzymes are fragile, leading to important considerations about increase storage and operational stability to enable their practical application. In this scenario, the use of nanoparticles to deliver proteins is increasing exponentially, given that these systems are capable of enhance active's stability, solubility and permeability, and decrease toxicity. In the pharmaceutical nanotechnology field, bromelain has played different roles and thus this paper aims to review the available literature for the use of nanoparticles and bromelain.

Key words: bromelain; nanoparticles; pharmaceutical nanotechnology

Introduction

Bromelain is a crude extract derived from pineapple plant (*Ananas comosus* L.) and contains mixture of proteolytic enzymes and non-enzymatic substances [1]. It can be found in several parts of the pineapple plant, including its stem, fruit, leaves and peel [2, 3]; only the stem and fruit, however, produce high amounts of bromelain [4, 5]. Stem bromelain (EC 3.4.22.32), found in the pineapple stem, has an isoelectric point (pI) of 9.5, and is the most abundant protease in pineapple tissue preparations. On the other hand, fruit bromelain (EC 3.4.22.33), which is found in the pineapple fruit, has a pI of 4.6, and is present in lesser amounts compared to stem bromelain [6, 7]. Stem bromelain is economical to produce once pineapple stems are cheap compared with the fruit, which is normally consumed. Therefore, stem bromelain, which is composed of endopeptidases (ananain, comosain), phosphatases, glucosidases, peroxidases, escharase, cellulases, glycoproteins, proteinase inhibitors, calcium and carbohydrate, is the most commonly available commercial product [1].

Like other cysteine proteases, the spectral characteristics of stem bromelain suggest that this enzyme belongs to the α+β protein class, with 23% α-helix, 5% parallel β-sheet, 18% anti-parallel β-sheet, 28% turns and rest other secondary structures [8, 9]. Stem bromelain contains 285 amino acids where the most abundant amino acids are alanine and glycine, while histidine and methionine are present in the lowest amounts [9-11], and its amino acid sequence has a high similarity to that of papain, actinidin, proteinase Ω and chymopapain [12].

Bromelain finds widespread applications in several areas, such as medicine, health, food, and cosmetics. Its applications have been widely reviewed in the literature: medical use [6], food industries and cosmetics [11], therapeutic applications [13], surgical care and related conditions [1], and bromelain commercialization for clinical and industry uses [5]. Literature shows that while the main application of bromelain continues to be in the pharmaceutical industry, the strong proteolytic activity of the enzyme supports its future application in many additional fields [13].

An increased storage and operational stability of an enzyme is an important consideration for its practical application [14], since most proteins and/or enzymes are fragile, and even small conformational changes may reduce their activity [15]. Several methods have been reported and used for enzyme stabilization, such as enzyme chemical modifications, protein engineering techniques, use of compatible osmolytes [14, 16]. In addition, the use of nanoparticles (NPs) to deliver protein drugs is increasing exponentially, once these systems stabilize those actives against denaturation by enzymatic digestion, increasing their biopharmaceutical applications [17].

Besides stability enhancement, nanostructures as drug delivery systems (nanocarriers) are a key to overcome other challenges associated with drug therapy, including poor solubility, poor permeability and high toxicity [18, 19]. Those nanometric carriers may also be used for the development of targeted delivery systems, which comprised a therapeutic agent, a targeting moiety, and a carrier system [20]. In the pharmaceutical nanotechnology field, bromelain has played different roles, such as surface modification [21-25], reducing and capping agent for gold NPs production [26-28], and immobilized or encapsulated active [29-36]. NPs have also been used as an alternative for bromelain purification [37-39] and quantification [40, 41].

The aim of this paper was to review the available literature for the use of NPs and bromelain in pharmaceutical area. For this purpose, Web of Science and PubMed databases have been used, crossing the terms “bromelain” and “nanoparticle”, resulting in 21 papers. Out of these, only 16 papers have been thoroughly revised according to the NP composition, excluding those 5 reporting bromelain purification and quantification.

1. Inorganic Compounds

1.1. Silica

Mesoporous silica nanoparticles (MSNs) are biocompatible materials, with large surface area and pore volume, providing great potential for drug absorption and loading within the pore channels [42]. MSNs mesoporous structure and adjustable pore size enable better control of drug loading and releasing [43]; and their surface can be easily modified for controlled and target drug delivery, enhancing drug therapeutic efficacy and reducing toxicity.

Parodi, Haddix [21] developed MSNs with a proteolytic (bromelain) surface, to enhance NPs diffusion features upon contact with tumor extracellular matrix. First non-modified MSNs were produced and characterized by TEM analysis, which showed a uniform size around 50 nm and spherical shape. Pore size and surface area were also analyzed, and found to be around 2.3 nm pore size and 650 m2/g of surface area to mass ratio [21]. Surface functionalization of MSN with bromelain was achieved and proven by FTIR spectroscopy. Size and zeta potential were determined for MSN and bromelain-conjugated MSN by DLS, and found to be 173.7 nm and 213.6 nm, +3.89 mV and -2.01 mV, respectively. The mass of bromelain linked to the MSN surface was quantified with Bradford protein assay [44], revealing 126.0 μg of protein extract/mg of unmodified particle was bound and absorbed after subtraction of the MSN background [21].

In this study, authors also showed that bromelain modification increased particles affinity for tumor extracellular matrix, and showed a minor impact on cell viability and cell

endolysosomal activity. Bromelain–MSN demonstrated efficient digestion and diffusion in matrigel, as well as inhibition of the organization of endothelial cells into tube-like structures when plated upon matrigel. Upon direct administration within the tumor, modified particles diffused to a greater extent within 1 hour than non-proteolytic MSN [21].

Silica supports are of particular interest for enzyme encapsulation in solid supports, due to their inert and stable matrix characteristics, with a pore size that can be tailored to the specific dimensions of the enzyme [45]. To enhance encapsulation efficiency, a bioinspired silicification technique has been used, wherein the enzymes are entrapped within silica nanosphere aggregates. Baker, Patwardhan [31] used sodium metasilicate as a silica precursor and ethyleneamines of different chain lengths as initiators to encapsulate papain, bromelain, and trypsin in silica nanosphere aggregates.

Overall, an encapsulation efficiency greater than 70.0% was observed for each protease, independent of ethyleneamine chain length, and thus pentaethylenehexamine (PEHA) was chosen as the initiator, due to its ability to provide rapid silica formation [31]. Morphology and particle size of the bioinspired silica aggregates were analyzed using field-emission scanning electron microscopy, showing smooth spherical particles with diameters ranging from 125.0 to 325.0 nm in the absence of proteases. Particles formed in the presence of either bromelain had morphology and size distribution similar to blank particles. Enzyme encapsulation was also confirmed by FTIR characterization and porosity measurements [31].

After encapsulated, hydrolysis and aminolysis were evaluated to determine if proteins remained their activities in comparison with free proteases. In casein assay, an activity reduction greater than 60.0% was observed when 2.0 mg of protein was used in the enzymes encapsulation process, and for bromelain only 12.1% of activity remained after encapsulation. This activity was partially recovered by increasing the total mass of the protease encapsulated in the silica aggregates from 2.0 mg to 10.0 mg, achieving 61.7% of remained activity for bromelain. To evaluate the effect in aminolysis, L-amino acid ethyl esters was used as substrate. Encapsulation did not change the percent yield of poly-L-leucine, but increased 10°C in optimal temperature for production of poly-L-leucine, which was found to be 40°C for free enzyme and 50°C for encapsulated form [31]. Thermal stability of free and encapsulated enzymes where also compared through enzymatic activity evaluation in temperatures ranging from 20 to 70 °C. Encapsulation process lead to an increase of 10°C in optimal thermal activity to bromelain. In addition, encapsulated proteases retained a higher relative hydrolytic activity then free enzymes at temperatures above that for optimal activity [31].

1.2. Gold

Gold NPs present various biomedical applications and present the possibility of surface functionalization, which has opened new perspectives for anticancer and antimicrobial drugs [46]. However, the most common methods for their production are hazardous to the environment despite their own disadvantages. Thus, the search for novel molecules for the synthesis of gold NPs have been motivated, and bromelain appeared as an alternative reducing as well as capping agent [26].

Khan, Danish Rizvi [26] adopted two different approaches to synthesize gold NPs of best qualities in terms of size, shape, monodispersity, and stability using bromelain. First, varying bromelain concentrations were incubated with a constant concentration of gold H[AuCl4] (1 mM) at constant temperature (40°C). NPs produced in this step were submitted to TEM analysis, which has shown the trend that the size of NPs increases gradually with the increase in bromelain concentration at 40°C. Thus, the optimum concentration of bromelain was determined as 0.33 mg/mL, at which NPs presented -16.60 mV zeta potential, hydrodynamic diameter of 58.7 nm and polydispersity of 0.239 by DLS technique, and were found to be monodisperse and spherical with size of 16.5 nm under SEM [26].

In the second approach, optimum bromelain concentration was kept constant and the temperature of incubation varied, as an attempt to increase formation rate of gold NPs. However, at higher temperatures, bigger size NPs were formed, lowering their stability [26]. Yet, with increase in temperature the activity of bromelain decreases gradually due to the degradation of protease [13, 47]. In conclusion, Khan, Danish Rizvi [26] study showed that gold NPs size and zeta potential depend on incubation temperature and bromelain concentration, and the optimum conditions to produce gold NPs using bromelain as reducing and capping agent was found to be 0.33 mg/mL concentration of bromelain at 40 °C. In addition, authors showed that synthesized NPs are highly stable, and thus they could be applied in other biomedical studies, such as the development of drug carriers [27, 28].

The main objective of Bagga, Ansari [27] was to develop bromelain capped gold NPs as effective drug delivery carriers of levofloxacin and to evaluate its antibacterial potential in comparison with pure antibiotic. For this, bromelain capped gold NPs were synthesized as described by Khan, Danish Rizvi [26] and were bio conjugated to levofloxacin using 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide as activator. Antibiotic loading efficiency was calculated using UV-visible spectroscopy, and found to be 84.8%, indicating that levofloxacin was efficiently loaded on synthesized NPs.

Both synthesized NPs were evaluated through DLS technique and the results showed that levofloxacin-bio conjugated NPs presented a higher hydrodynamic size than unconjugated particles, which were 58.7 and 38.1 nm respectively. Size was also analyzed by TEM and found to be around 13.2 nm for bio conjugated NPs and 11.4 nm for unconjugated. The zeta potential of NPs with levofloxacin was found to be -9.01 mV, while bromelain capped gold NP presented a potential of -13.80 mV, which supports the absence of NP aggregation after conjugation. This change in zeta potential may be due to the decrease in the number of carboxylic groups, once they are involved in conjugation of levofloxacin as it was showed by FTIR spectrum. Observed sized difference between conjugated and unconjugated NPs, as well as difference in the zeta potential can serve as an evidence that the conjugation has taken place, and supports the absence of aggregation of NPs after bio conjugation [27].

Antibacterial efficacy of levofloxacin-bioconjugated NPs and pure levofloxacin was determined by evaluating MIC against *S. aureus* and *E. coli*, and percent inhibition of both bacteria occurred in dose-dependent manner for both samples. The functionalized NPs showed superior antibacterial activity compared to pure levofloxacin at the similar concentration, presenting an IC_{50} values reduction from 0.547 µg/ml and 1.96 µg/ml for pure antibiotic to 0.128 µg/ml and 1.10 µg/ml of NPs, against *S. aureus* and *E. coli* respectively. Authors attributed these results to NPs superior stability and the transport of a huge number of levofloxacin molecules into a highly localized area [27].

Shaikh, Rizvi [28] prepared a nanoformulation of cefotaxime using gold NPs to combat drug-resistance in β-lactamases having the expanded spectrum producing strains. In this study, gold NPs were also produced using bromelain as reducing and capping agent [26], and after cefotaxime was conjugated with NPs using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide activator. SEM analysis showed that both produced NPs were of spherical shape and monodispersed. Unconjugated and cefotaxime-conjugated NPs presented an average size of 6.9 and 17.6 nm, respectively, using TEM analysis, and an average size of 11.8 and 29.7 nm, respectively, by DLS. These results suggested that there was an increase in gold NP size owing to drug attachment.

Antibacterial efficacy against *E. coli* and *K. pneumonia* resistant strains was estimated using agar well diffusion, MIC and MBC assays. In the agar well diffusion, both strains were completely resistant to cefotaxime alone at 3.23 and 6.46 mg/L concentrations of cefotaxime, while zone of inhibitions of cefotaxime-conjugated NPs *E. coli* were 14 and 18 mm, respectively; and against *K. pneumoniae* were 13 and 17 mm, respectively, using the same concentration as free cefotaxime. MIC of cefotaxime-conjugated NPs was found as 1.009 and

2.018 mg/L against the study strains of *E. coli* and *K. pneumoniae*, respectively. Whereas, MBC of cefotaxime-conjugated NPs were determined to be 2.018 and 4.037 mg/L for the same strains, respectively. Unconjugated NPs were also tested as control and were inactive against the tested drug resistant strains [28].

2. Synthetic Polymers

2.1. Poly(acrylic acid)

Poly(acrylic acid) (PAA) is a biocompatible hydrophilic polymer, which can easily be modified chemically through the free carboxyl groups [48], and many attempts have been made to create particulate systems based on PAA [49].

Some other studies aimed the preparation of modified PAA using carbodiimide technique, and for example papain and bromelain were conjugated with PAA NPs to overcome intestinal mucus gel layer [23]. Bromelain were firstly covalently conjugated via carbodiimide chemistry to PAA, and the resulting polymers were dialyzed and lyophilized, yielding white powders. Conjugation process was successful, with a product recovery of 59.2%. However, enzyme conjugation onto the polymeric scaffold provoked a loss of enzyme activity, when compared to the native bromelain activity, and the residual enzymatic activity was found to be 63.3% [23].

NPs were then formulated via ionic gelation method exploiting Ca^{2+} as counter ion, using PAA or enzyme-PAA polymer. Enzyme-conjugated and unmodified NPs were monodispersed, with an average diameter around 285.0 nm and 234.0 nm for bromelain and non-modified PAA, respectively. The zeta potential resulted to be negative for all formulations with slightly higher values for the enzyme-modified NPs, being around -9.40 mV, and -5.10 mV for non-modified PAA and bromelain, respectively. The enzyme content in the modified NPs were assessed after treatment with sodium dodecyl sulfate leading to particles disaggregation, and found to be 253.0 µg enzyme/mg for bromelain; also NPs were able to maintain 76.0% of enzymatic activity. Thereby authors conclude that NPs could be formulated with these modified polymers, presenting similar physical-chemical properties in terms of particles size, zeta potential and enzyme content [23].

NPs permeation ability was investigated by the rotating tube technique, providing data about the depth of NPs diffusion into porcine mucus. Bromelain modified NPs could permeate until the last mucus segments (segment 9), while non-modified PAA NPs were detected only in the five first segments, indicating that enzymatic modification enhanced particles permeation through mucus barrier. NPs interaction and effect on the mucus barrier was also confirmed by

pulsed-gradient spin-echo NMR, showing that enzyme conjugated NPs lead to an increase in mucin diffusivity. Therefore, authors concluded that bromelain showed potential to improve the permeability of PAA NPs, and can be exploited as a drug carrier able to transport therapeutic molecules through the mucus barrier and consequently able to improve the bioavailability of orally administered drugs [23].

Wilcox, Van Rooij [24] evaluated the effect of bromelain modified PAA NPs and non-modified PLGA NPs on the bulk rheology properties of porcine intestinal mucus from the small intestine. Bromelain modified PAA NPs were produced via ionic gelation method, presenting an average size 284.6 nm, polydispersity index of 0.200, and zeta potential around -5.09 mV, with 253.4 µg enzyme/mg NPs, and residual enzymatic activity of 76.0% as previously described by Pereira de Sousa, Cattoz [23]. In turn, the PLGA NPs were produced by nanoprecipitation method, and showed an average size around 136.0 nm, polydispersity index of 0.030, and zeta potential -23.06 mV, as previously described by Khayata, Abdelwahed [50]. Porcine intestinal mucus was incubated in the presence of PLGA NPs or PAA NPs decorated with bromelain, and the effect of NPs on the rheological properties, weight of gel, released glycoprotein content from mucus as well as the viscosity of liquid removed was assessed [24].

PLGA NPs were known to permeate into mucus, although in low amounts, and proteolytic enzymes-decorating NPs has been shown to increase the permeation through mucus [23, 24]. Thus, study results are in accordance to literature, once treatment with NPs decreased mucus gel strength, with bromelain-modified particles reducing it the most. Modified NPs also resulted in the highest glycoprotein release (11.2 mg/mL) from gel network, when compared to PLGA NPs (3.6 mg/mL) and intestinal fluid (3.2 mg/mL), whereas mucus treated with proteolytic-modified particles remained a gel and exhibited a similar breakdown stress to control mucus samples. Therefore, authors concluded it would be possible to use bromelain to increase the permeability of NPs through mucus without destroying the gel and leaving the underlying mucosa unprotected [24].

2.2. Poly(lactide-co-glycolide) acid

Poly(lactide-co-glycolide) acid (PLGA), a copolymer of lactic and glycolic acid, is one of the most widely used synthetic polymer for biomolecules encapsulation. The well-documented biocompatibility and safety of these materials, as well as their biodegradability and controlled release kinetics have led to their FDA approval. The most commonly used method for the preparation of PLGA NPs is the double emulsion-solvent evaporation method using dichloromethane or ethyl acetate as the polymer solvent [18].

PLGA NPs were then functionalized with the proteolytic enzymes trypsin, papain and bromelain via a two-step carbodiimide method, with different enzyme:NPs mass ratios to investigate enzyme concentration effect on the enzyme conjugation efficiency and particles physicochemical properties. Results showed that an increase in the initial enzyme:NPs mass ratio leads to an increase of the particle enzyme loading, achieving up to 5.34% of loading efficiency for bromelain. Functionalized NPs had an average diameter of 300.3 nm to 306.4 nm and a negative zeta potential value of -21.80 to -29.40 mV, depending on enzyme:NPs mass ratio [22].

After conjugation with NPs, there was a decrease in the enzymatic activity, which was attributed to coupling reaction and the concomitant conformational changes in enzyme structure. Even though conjugated enzyme maintained a significant residual enzyme activity percentage of 27.6% for bromelain. Authors also demonstrated that functionalized NPs were stable within two weeks' time, presenting no change in particle size distribution and zeta potential [22].

According to the reported results, surface functionalization with proteolytic enzymes largely increases PLGA NPs mucus permeability, and bromelain showed a three-fold higher permeability in porcine intestinal mucus compared to non-modified NPs. Those observations were further confirmed using nuclear magnetic resonance technique, as the enzyme-functionalized NPs were proven to be capable of disrupting the mucin gel structure [22].

Bhatnagar, Patnaik [32] encapsulated bromelain in PLGA NPs by water-in-oil-in-water double emulsion solvent evaporation method, with further NPs coating with Eudragit L30D polymer to introduce stability against the gastric acidic conditions. Developed NPs showed an average size around 147.9 nm, with 0.13 of polydispersity index, and a zeta potential of -9.16 mV using DLS characterization. TEM results revealed that the NPs had distinct spherical shape with mono-disperse in nature and a size raging between 25-45 nm. For the coated NPs, authors report an entrapment efficiency of 48.0% and a drug loading of 4.5%. Bromelain proteolytic activity was measured using casein, and found to be 698.2 units/mg and 673.9 units/mg for free and encapsulated bromelain, respectively, showing that bromelain was kept bioactive and stable after the encapsulation process [32].

Bromelain release from Eudragit coated-PLGA NPs was investigated in PBS at pH 7.4 and temperature 37°C for 9 days, and an initial burst release of bromelain occurred in the first 6 hours, releasing up to 15.0% of loaded bromelain. Afterwards, bromelain release was sustained and continued over a period of 8 days, reaching 89.0%, and data showed that the formulation followed Higuchi's release model, as R^2 reached 0.9 value [32]. Since Eudragit

was used to introduce stability in gastric conditions, bromelain release from Eudragit-coated and non-coated NPs was also investigated in acidic conditions (pH 1.5) and it was around 28.0% after 24 hours for non-coated NPs, whereas it was only 10.0% after 24 hours for coated NPs, evidencing the protection of Eudragit.

NPs hydrodynamic diameter and PDI did not significantly change when NPs were incubated in various pH mediums (pH 1.5, 3.5, and 5.0), suggesting NPs stability under these conditions. Free and encapsulated bromelain were also submitted to *in vitro* cytotoxicity assays and *in vivo* tests using Ehrlich ascites carcinoma bearing Swiss albino mice. *In vitro,* NPs significantly reduced the IC_{50} values compared with free bromelain, while NPs showed enhanced anticancer activity *in vivo*, with significant decrease in tumor burden on mice [32].

In a subsequent study, after synthesis and characterization, Bhatnagar, Pant [33] used bromelain-PLGA NPs as an anti-carcinogenic agent against 7,12-dimethylbenz[a]anthracene induced skin cancer in mouse model, comparing with free bromelain. Bromelain-PLGA NPs were produced in the same way as previously reported [32], and the resulting NPs had similar characteristics: 52.0% of entrapment efficiency and drug loading of 4.7%; mean particle size of 130.4 nm and 0.095 of PDI by DLS; smooth and spherical morphology and particle size in the range of 20–35 nm by TEM analysis. A burst release of bromelain was observed from the NPs formulation, reaching 17.0% and 21.0% of the encapsulated bromelain at pH 7.4 and 6.5, respectively, in the initial 12 hours. After this, NPs showed a sustained release of bromelain, which was faster in acidic pH: 90.0% of the encapsulated bromelain was released after 5 days at pH 6.5, while 87.0% was released after 8 days at pH 7.4. During colloidal stability assay, NPs did not present statistical difference in hydrodynamic diameter at both pH values, rendering the NPs stable [33].

Bromelain proteolytic activity was measured in casein digesting units and found to be 564.0 and 525.0 units/mg for free bromelain and extracted bromelain from PLGA NPs, respectively. Once again, the results showed an insignificant decrease in proteolytic activity of bromelain encapsulated in PLGA NPs. Encapsulated bromelain revealed enhanced anti-tumor ability in 2-stage skin tumorigenesis mice model, presenting a reduction in average number of tumors, delay in tumorigenesis, and percent mortality rate as well as a reduction in the average tumor volume when comparing with free bromelain. NPs were found to be superior in exerting chemopreventive effects over chemotherapeutic effects, demonstrated by the enhanced ability of NPs to protect the DNA from induced damage, which was supported by histopathological evaluations. NPs were also capable of modulating the expression of pro-apoptotic and anti-apoptotic proteins [33].

Continuing the studies, Bhatnagar, Pant [35] developed hyaluronic acid (HA) grafted PLGA copolymer, having tumor targeting ability, and then encapsulated bromelain in copolymer to obtain bromelain-copolymer NPs, using different bromelain to polymer weight ratios. Bromelain:polymer weight ratios interfere NPs mean size, PDI, encapsulation efficiency and drug loading, and the best parameters were found when using 1:15 feed ratio. Using 1:15 ratio, NPs mean size was 146.3 nm, with 0.340 of PDI, 85.8% of encapsulation efficiency, 5.3% of drug loading. TEM analysis showed an almost monodispersed NPs formation, with size between 30–40 nm, while SEM images showed the NPs typical spherical morphology with distinct smooth surfaces. An initial burst release of 21.7% of bromelain from copolymer NPs in the initial 12 hours was observed, followed by a continuous release of bromelain amounting about 90.3% of bromelain in 5 days. Bromelain proteolytic activity was evaluated by casein digestive units and found to be 698.2 and 680.7 units/mg for free and encapsulated bromelain, respectively, did not showing any statistically significant difference [35].

Developed bromelain-copolymer NPs were delivered efficiently to the various cancer cells, but largely in those with high CD44 receptors, resulting to a higher cytotoxicity. It was also observed that the cytotoxicity of NPs was time dependent, indicating more significant cytotoxicity after 48 and 72 hours of incubation as compared to 24 hours, which authors speculated to be due to small amount of bromelain released from the NPs in initial 24 hours. The *in vivo* antitumor efficacy of free and encapsulated bromelain, administered via intraperitoneal and intravenous routes was evaluated in Ehrlich Ascites Carcinoma bearing Swiss albino mice and results showed that NPs were efficient in suppressing the tumor growth [35].

3. Natural Polymers

3.1. Chitosan

Chitosan is a mucopolysacharide, obtained by deacetylation of chitin, the major compound of exoskeletons in crustaceans [51]. Different methods can be employed to produce chitosan NPs, i.e. emulsion, ionic gelation, reverse micellar and self-assembling methods [52, 53], and particle size, particle formation, and aggregation are directly affected by the molecular weight and degree of deacetylation [51].

Different chitosan derivatives have been synthesized and studied for their potential applications. Modification does not result in change of the chitosan fundamental skeleton but brings derivatives characterized by new or improved properties [54]. Carboxymethyl-chitosan

(CMCS) is a negatively charged derivate of chitosan, which was developed to improve chitosan solubility, have been extensively applied in a wide range of biomedical field as a drug carrier.

Linoleic acid can be covalently conjugated to carboxymethyl-chitosan (CMCS) via a 1-ethyl-3-(3-dimethylaminopropyyl)-carbodiimide-mediated reaction, generating self-aggregated chitosan NPs by sonication, with an average hydrodynamic diameter around 417.8 nm [55]. Bromelain immobilization onto these NPs was studied by the addition of varying enzyme concentrations (from 0.05 to 0.4 mg/mL) to 1 mL of NPs. Results showed that bromelain loading efficiency was affected by its concentration: as the concentration of bromelain increased, the loading efficiency decreased. On the other hand, the bromelain loading capacity was enhanced by increasing enzyme concentration, and NPs were almost saturated by bromelain at 0.3 mg/mL concentration [29].

In this study, authors investigated factors affecting the enzymatic activity of immobilized bromelain, including temperature and storage. Enzyme optimal temperature was determined carrying out the enzyme assay at different temperatures (from 20°C to 80°C) at pH 7.4, and it was founded that both, free and encapsulated bromelain, had the same optimal temperature (60°C). Enzyme thermal stability was also evaluated and relative activities of both free and immobilized enzymes decreased with temperature increase; however, less activity was lost for immobilized bromelain at every tested temperature compared to free enzyme. The improve in bromelain NPs thermal stability was attributed to the multiple interactions between bromelain and NPs, and to its entrapment into NPs' core, which could protect the enzymatic configuration from distortion or damage by heat exchange [29].

The Michaelis constant (Km), which corresponds to the substrate concentration resulting in half of the maximum reaction velocity, was calculated and used to evaluate the ability of forming complex between enzyme and substrate. Obtained results showed that both immobilized and free enzyme exhibited almost the same curves, indicating the complex formed by bromelain and NPs rarely affected interaction of enzyme and substrate. The apparent Km of immobilized enzyme was 0.36, smaller than that of free enzyme, 0.68, which indicates that encapsulation enhanced enzyme affinity for substrate. Authors attributed this result to mild immobilization process, which slightly affected enzyme's structural or conformational integrity, and to electrostatic or hydrophobic interactions between NPs and casein, which may strengthen enzyme affinity for casein substrate [29].

Lactobionic acid (LA), a tumor-homing ligand was reacted with the amino groups of chitosan oligosaccharide, to give tumor-targeted polymer: lactobionic acid-modified chitosan (CLA). Recently, bromelain was used to improve the tumor penetration ability of CLA NPs

[25]. CLA NPs, with the diameter ranging from 150.0 nm to 250.0 nm, were obtained by desolvation method and varying the reaction conditions. Bromelain-modified and non-modified CLA NPs had hydrodynamic particle size of 243.5 nm and 197.5 nm, respectively, determinate by DLS; they were in spherical shape with a homogeneous size of 150 nm by TEM and SEM analysis [25].

Enzymatic activity of bromelain-modified CLA NPs on gelatin degradation was measured. For this test, gelatin was dissolved in warn water, co-incubated with free bromelain, non-modified CLA NPs and bromelain-modified CLA NPs, and then stored at 4°C. It was possible to observe that pure gelatin solution and non-modified CLA NPs co-incubated solution were able to form a steady gel, while the free bromelain and bromelain-modified CLA NPs co-incubated gelatin solution still maintained in liquid state, which indicate gelatin degradation into low molecular weight products. Thus, it demonstrated that CLA NPs surface was successfully decorated with bromelain, which still kept its enzymatic activity [25].

In this study, doxorubicin was used as a model anticancer drug and loaded into these prepared NPs, using different NPs:doxorubicin mass ratios (10:1, 7.5:1, 5:1 and 3:1). CLA NPs loaded with doxorubicin had an average size ranging from 201.0 nm to 231.0 nm with drug loading content from 7.8% to 31.1%, depending on the used mass ratio. Bromelain-modified CLA NPs average size and drug loading content was also mass ratio-dependent, ranging from 24.01 nm to 307.0 nm, and 6.3% to 33.2%, respectively. Bromelain conjugation did not influence on doxorubicin release, since its profile was similar from non-modified NPs. The lowest cumulative release was observed at pH 7.4, with 13.1% and 18.1% of doxorubicin released from non-modified CLA NPs and bromelain-modified NPs, respectively; while the highest release occurred at pH 5.5, with 72.4% and 76.3% release, respectively. The release profile results showed that both NP carriers could continuously release drug at acid environment, making them suitable for tumor drug delivery [25].

In vitro cellular uptake and flow cytometry methods, using HepG2 and SH-SY5Y cells, were used to investigate accumulation and distribution of doxorubicin in its free and encapsulated in bromelain-modified and bon-modified CLA NPs. Results demonstrated that HepG2 cell internalized both NPs more efficiently than free doxorubicin. On the other hand, SH-SY5Y cells were more efficiently in internalized free doxorubicin than both NPs. Both NPs loaded with doxorubicin showed higher cytotoxicity toward HepG2 than SH-SY5Y cells, which is consistent with the results of cellular uptake [25].

3.2. Katira gum

Katira gum, a natural anionic polysaccharide isolated from *Cochlospermum religiosum*, is non-toxic and insoluble and has gained importance due to its versatile applicability in food industries and its pharmacological usability [56], being described as a novel suitable pharmaceutical excipient for formulation development. Further, because of its stability in terms of rheology and microbial flora, it has been proposed as a potential novel drug delivery candidate [34].

Bromelain-loaded katira gum NPs were prepared by ionotropic gelation method employing calcium chloride or magnesium chloride as cross-linkers, and the preparation was optimized using response surface methodology of central composite design. In the experimental design, type of cross linker and concentration of katira gum, cross linker and bromelain were chosen as formulation variables, whereas particle size and zeta potential were used as response variables. Bromelain encapsulation efficiency varied between 39.6 and 80% in the various batches of NPs, however this the response did not fit into a polynomial model. The optimized batch, composed of 0.87% (w/v) katira gum, 0.1% (w/v) calcium chloride, and 16.82% (w/w of katira gum) bromelain, produced NPs with 156.2 nm of particle size, -26.1 mV of zeta potential, and entrapped about 70.0% of the added bromelain [34].

This optimized NP formulation was submitted to other characterization assays. FTIR spectroscopy indicate no chemical interaction between bromelain and katira gum. Bromelain-loaded katira gum NPs were observed to be spherical in shape when viewed under TEM and showed a particle size of 10-13 nm. Anti-inflammatory activity of bromelain loaded in katira gum NPs was evaluated in carrageenan-induced rat paw edema model, and rats were orally treated. *In vivo* results showed that increasing bromelain dose led to a significant inhibition in paw edema. When comparing bromelain-loaded NPs with bromelain, a higher edema inhibition was observed for NP formulation, and no effect was observed for unloaded katira gum NPs, indicating that the enhanced anti-inflammatory activity of bromelain-loaded katira gum NPs cannot be attributed to synergistic effect of polymer and bromelain [34].

4. Niosomes

Liposomes are phospholipid vesicles containing one or more bilayers enclosing an aqueous core [57], and they can be divided into two broad categories as conventional and novel liposomes. Conventional liposomes are mainly composed of pure or mixtures of phospholipid(s) and may or may not contain cholesterol. On the other hand, novel liposomes

include a series of different liposomal systems containing other actives apart from phospholipids, yielding deformability or elasticity to the liposomal bilayers [58].

Several studies report the development of novel elastic vesicles in order to deeply and easily penetrate through the skin, and among them, the elastic niosomes are the most recent novel elastic nanovesicles which have been developed [59, 60]. This developed elastic niosomal formulation was used for protease enzymes (papain and bromelain) incorporation, aiming keloids and hypertrophic scars treatment. The 5% sodium cholate elastic niosomes were prepared by the thin film hydration method with sonication [59], and enzymes-loaded niosomes were prepared by hydrating the thin film with enzymes solutions in 5 mM PBS (pH 7.0) at various concentrations (from 0.4 to 5.0 mg/mL). Ten different niosomal dispersion were developed, and all of them were in translucent colloidal appearances, with no sedimentation or layer separation [30].

Blank and enzymes-loaded elastic niosomes gave smaller sizes than those of the non-elastic niosomes [59]. Bromelain-loaded niosomes presented a smaller particle size than blank particles, an effect that was attributed to charge interaction between bromelain and vesicular membrane, once at pH 7.0 bromelain (pI = 4.6) is negatively charged and can only be loaded in the niosomes. Non-elastic and elastic niosomes loaded with extracted bromelain gave higher vesicular sizes than those loaded with the standard, an effect that was attributed to impurities contained in extracted bromelain, such as comosain and ananain (pI > 8.5), which were positively charges in pH 7.0 and could bound on the niosomal membrane by ionic interaction, resulting in larger vesicular size than those of the standard bromelain. Elastic niosomes bromelain-loaded showed superior elastic property and entrapment efficiency than non-elastic niosomes. Due to charge interaction and impurities presence in extracted bromelain, the entrapment efficiency of extracted bromelain was higher than the standard bromelain in elastic niosomes, and found to be around 37.4% and 34.9%, respectively [30].

Human skin fibroblasts viability was evaluated using sulforhodamine B assay by exposing cells to various concentrations of free and encapsulated enzymes, as well as blank niosomes. The blank elastic niosomes even at the concentration of 0.5 mM did not showed cytotoxicity with cell viability of more than 95.0%. Free and loaded bromelain effect on fibroblasts viability was dose dependent and as the concentration increased, the viability decreased. Both papain and bromelain loaded in elastic niosomes gave no cytotoxicity, with cell viability of more than 80.0%. At 0.1 mg/mL concentration, standard and extracted bromelain loaded elastic niosomes showed 94.2% and 82.7%, respectively, which were 1.56 and 1.52 times more than their corresponding free enzyme. In addition, both the relative pro

MMP-2 and active MMP-2 of both standard and extracted enzymes (papain and bromelain) loaded in elastic niosomes were slightly decreased, but no significant difference was observed when comparing to free enzymes [30].

5. Lipid Core Nanocapsules

Nanocapsules can be defined as nano-vesicular systems that exhibit a typical core-shell structure in which the drug is confined to a reservoir or within a cavity surrounded by a polymer membrane or coating, and stabilized by surfactants [61]. This reservoir can be lipophilic or hydrophobic according to the preparation method and raw materials used. Among the nanocapsules formulations, a new kind, named lipid-core nanocapsules (LCNs), which are composed by a dispersion of sorbitan monostearate and medium chain triacylglycerol, in the core, enveloped by poly(ε-caprolactone) (PCL), an aliphatic polyester as polymeric wall have been reported [62]. Oliveira, Prado [36] proposed the development of bromelain-surface functionalized LCNs, aiming the formulation applicability to improve the antiproliferative effect of bromelain against cell cultures of human breast cancer (MCF-7).

Initially to produce bromelain-functionalized chitosan-lecithin-polysorbate 80-coated lipid-core nanocapsules, a pre-formulation study to optimize the concentrations of lecithin and chitosan in the formulations was carried out. Three different concentrations of soybean lecithin and eight different concentrations of chitosan were tested, and from those formulations, only four showed unimodal size distribution by laser diffraction analysis. The chosen formulation was that with higher zeta potential (around +26.00 mV), containing 6 mg/mL of lecithin and 0.1% (w/v) of chitosan concentration. Following, a pre-formulation study was also conducted to find the proper proportion between the metal ion (Zn^{2+}) and bromelain, where six different formulations were prepared and their size distribution profiles by laser diffraction and zeta potential were analyzed. The chosen formulation was prepared using 50 mg/mL of bromelain and 50 mg/mL of Zn^{2+}, and had an average diameter around 135.0 nm, PDI of 0.180, and zeta potential of +23.00 mV. Formulation parameters were monitored for 10 days in order to determine particles stability and no significant difference was observed [36].

Bromelain proteolytic activity was determined using casein as substrate for three different samples: bromelain solution, Zn-bromelain solution and bromelain-functionalized LCNs. Bromelain in contact with Zn^{2+} maintained its proteolytic activity (around 0.12 U/mL); however, the activity was significantly lower than that of the free bromelain (around 0.22 U/mL). Bromelain-functionalized LCNs showed a specific activity around 0.054 U/mg in 30 minutes and 0.114 U/mg in 6.5 hours, which was similar to that of Zn-bromelain solution (0.020

and 0.117 U/mg in 30 minutes and 6.5 hours, respectively), suggesting that bromelain is complexed with Zn^{2+} in the nanocapsules formulation. During *in vitro* MCF-7 cell viability assays, nanocapsules formulations without bromelain functionalization presented similar results to those presented by control. Bromelain-functionalized LCNs treatment showed lower cell viabilities when compared to both bromelain solutions (with or without Zn^{2+}), as well as to the control group, being capable of decreasing MCF-7 cell viability in more than 35.0% when applied at 0.625 µg/mL [36].

Conclusions and Future Perspectives

We have reviewed the literature involving bromelain-loaded nanoparticles. Although bromelain has different applications in the pharmaceutical/medical fields, its delivery and use has been a challenge once enzymes have limited stability, being susceptible to denaturation and conformational changes that may reduce their activity. Nanotechnology has overcome some limitations of conventional delivery, such as poor water solubility, limited bioavailability due to poor absorption or stability issues. Bromelain immobilization or encapsulation has proven to be a good alternative to enhance bromelain enzymatic activity, broadening its pharmacological applications.

Beyond that bromelain has successfully played different roles in the pharmaceutical nanotechnology field, modifying nanoparticles surface, and serving as reducing and capping agent for nanoparticles production. In all its application together with nanotechnology, bromelain has significantly added its properties and activities, leading to better formulations outcomes. Still, there is not a unanimous nanoparticulated system for bromelain, once the subject is in its infancy. It is also important to highlight that in accordance with intended usage, *in vitro* and *in vivo* toxicity and release studies will be needed.

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CAPÍTULO IV. "BROMELAIN-CHITOSAN NANOPARTICLES: EFFECT OF POLYSACCHARIDE SOURCES ON THE PHYSICOCHEMICAL PROPERTIES OF NANOPARTICLES"

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Abstract

Nanoparticles present desirable characteristics as protection of active against degradation, being widely used for the development of drug delivery systems. Chitosan-based nanoparticles receives special attention due to its characteristics as mucoadhesion, availability, biocompatibility and biodegradability, and due to the variety of production methods. Bromelain, a set of proteolytic enzymes found in Bromeliaceae family with potential pharmaceutical properties, is unstable when directly applied in topical formulations, which limits its use. Therefore, this study aims to encapsulate bromelain in chitosan nanoparticles to enhance enzyme stability, testing chitosan from different sources and modifications, i.e. low molecular weight chitosan, chitosan oligosaccharide lactate, and chitosan from shrimp shells. Chitosan-bromelain nanoparticles were produced by ionic crosslinking technique, using sodium tripolyphosphate as crosslink agent. Produced nanoparticles were characterized by dynamic light scattering, zeta potential, nanoparticles tracking analyses, encapsulation efficiency, scanning electron microscopy and Fourier transformed infrared spectroscopy. Physicochemical and morphological characterization allows to confirm nanoparticles formation, and bromelain encapsulation higher than 84% and 79% for protein content and enzymatic activity, respectively. Nanoparticles suspension were also tested for accelerate stability and rheological behavior. It was not possible to obtain conclusive results in accelerated stability test, due to samples' high transmittance; and in general, bromelain addition decreased cohesiveness of nanoparticles suspension.

Keywords: bromelain; chitosan nanoparticles; physicochemical characterization; low molecular weight chitosan; chitosan oligosaccharide lactate; chitosan from shrimp shells

1. Introduction

Nanoparticulated systems have been widely used for different applications, mainly for drug delivery (Teimouri et al., 2018), presenting desirable characteristics such as modified release, localized retention and active protection against degradation (Mohammed et al., 2017). Polymeric nanoparticles gained special attention as drug delivery systems due to their biocompatibility and biodegradability, and the availability of their production methods (Bhattarai et al., 2006; Mohammed et al., 2017). Chitosan-based nanoparticles have been widely used because of chitosan's availability, biocompatibility, biodegradability, mucoadhesion, easy surface modulation, and low toxicity (Bhattarai et al., 2006; Mohammed et al., 2017). In addition, chitosan exhibits antibacterial, antifungal and antitumor activities, haemostatic properties, accelerates wound healing and stimulates the immune system (Severino et al., 2017; Teimouri et al., 2018; Teixeira et al., 2017; Vivek et al., 2013).

Chitosan is obtained by deacetylation of chitin, which is derived from crustacean and shells, and fungi cell walls, being one of the most abundant natural polysaccharide (Barbosa et al., 2016; Hasanifard et al., 2017; Mohammed et al., 2017; Severino et al., 2016). Reaction conditions and extent and chitin source are factors that can be modified during chitin deacetylation process. These modifications alter chitosan final characteristics, such as molecular weight, pKa and deacetylation degree, changing chitosan's physical and chemical properties (Mohammed et al., 2017; Wang et al., 2011).

Chitosan nanoparticles have been used as carrier for genes, proteins, vaccines, and antiallergic, antiviral, and anticancer drugs, through various administration routes, as oral, intravenous, nasal, vaginal and ocular (Ferreira da Silva et al., 2015; Hasanifard et al., 2017; Jose et al., 2011; Severino et al., 2014; Severino et al., 2012; Wang et al., 2011). Proteins are easily degraded in vivo by enzymes, having poor stability and short half-life, as well as poor permeability; and chitosan nanoparticles can act protecting protein, promoting contact between protein and cell membrane and modifying protein release (Jose et al., 2012, 2013; Wang et al., 2011).

Bromelain is a set of proteolytic enzymes found in Bromeliaceae family, mainly in pineapple (*Ananas comosus* L.), with potential pharmaceutical properties such as anti-inflammatory, antithrombotic, fibrinolytic, antitumor activity and immunomodulatory effect (de Lencastre Novaes et al., 2016; Rathnavelu et al., 2016; Taussig & Batkin, 1988). Bromelain has been investigated in wound healing and as debridement agent (Ataide et al., 2018; Muhammad & Ahmad, 2017; Romano et al., 2014). Previous studies showed bromelain instability when directly applied as topical formulations, even when stored at low temperatures

(Lourenço et al., 2016; Pereira et al., 2014; Spir et al., 2015), pointing out that bromelain wound benefit from chitosan-based encapsulation. This study aims therefore to encapsulate bromelain in chitosan nanoparticles, testing chitosan from different sources and modifications.

2. Materials and Methods

2.1. Materials

Bromelain from the pineapple stem, azocasein, Bradford reagent, low molecular weight chitosan (catalog number 448869), chitosan from shrimp shells (catalog number 50494) and chitosan oligosaccharide lactate (catalog number 523682) were purchased from Sigma-Aldrich® (St Louis, EUA). All the other reagents were purchased in analytical grade.

2.2. Standard solution of bromelain

Bromelain standard solution (10mg/mL) was prepared by dissolving bromelain in distilled water and filtered using 0.22-µm membrane.

2.2.1. Protein concentration and enzymatic activity

Total protein concentration was determinate following the method described by Bradford (1976). For bromelain enzymatic activity measurement, azocasein was used as substrate (Coelho et al., 2016; Sarath et al., 1989), at 37°C for 10 minutes. After this, reaction was interrupted by trichloroacetic acid addition. Mixture was centrifuged and absorbance of a supernatant aliquot (200µL) was measured at 440nm in microplate reader (Synergy™ HT, BioTek Instruments Inc., EUA). Enzymatic activity was then calculated in activity units (U/mL), which is the amount that causes increase of one unit in absorbance of 1 mL of sample in 60 minutes.

2.3. Nanoparticles production with different chitosan types

Nanoparticles were produced by ionic crosslinking technique (Severino et al., 2016), using sodium tripolyphosphate (TPP) as crosslinking agent, in 30% (w/w) concentration ratio of TPP to total chitosan amount, and mechanical stirring at approximately 350 rpm (Multistirrer 15 Magnetic Stirrer, Velp Scientific Inc., USA). In general, for each 2 mL of chitosan solution at 2.5 mg/mL (previously filtered on 0.45 µm membranes), 3 mL of TPP solution at 0.5 mg/mL (filtered at 0.22 µm) were dropwise added. Immediately after the TPP addition, 1mL of standard bromelain solution was added to produce chitosan-bromelain nanoparticles, or 1mL of distilled water to produce chitosan nanoparticles.

In order to compare different specifications and origin, three chitosan types were used for nanoparticles production, i.e. low molecular weight chitosan (LMW), chitosan oligosaccharide lactate (LAC), and chitosan from shrimp shells (SHR).

2.4. Nanoparticles characterization

2.4.1. Dynamic light scattering (DLS) and zeta potential

Size distribution, polydispersity and zeta potential of produced nanoparticles were evaluated using Zetasizer Nano ZS equipment (Malvern Instruments, Malvern, UK). The average size and polydispersity index were determined by dynamic light scattering (DLS), while zeta potential was determined using laser Doppler microelectrophoresis at 25°C (Bhattacharjee, 2016).

2.4.2. Nanoparticles tracking analysis (NTA)

Nanoparticles tracking analysis (NTA) was also used to determine nanoparticles mean diameter and size distribution, using NanoSight NS300 (Malvern, United Kingdom) equipment. Technique enable the determination of nanoparticles concentration expressed as number of particles/mL. Prior to analysis, nanoparticles suspensions were diluted 1000 times in Milli-Q water.

2.4.3. Encapsulation efficiency

To determine bromelain encapsulation efficiency, nanoparticles were centrifuged (Centrifuge 5810R, Eppendorf, Germany) for 10 minutes at 14.000g using 0.5 mL ultrafiltration devices with a 100kDa membrane (Amicon® Ultra 100k, Millipore, Germany). Total protein concentration and enzymatic activity were determined, as previously described, in initial bromelain solution, as well as in filtrate and withheld liquids, and in nanoparticles suspensions with and without bromelain. The encapsulation efficiency was determined by the difference in protein concentration and enzymatic activity in the initial bromelain solution and in the filtered solution, according to equation 1.

$$\text{EE (\%)} = \frac{Initial\ bromelain - Filtered\ bromelain}{Initial\ bromelain} \text{x100} \qquad \text{Equation 1}$$

2.4.4. Scanning electron microscopy (SEM)

Nanoparticle morphological characteristics were observed using a scanning electron microscope LEO 440i with X-ray dispersive energy detector 6070 (LEO Electron Microscopy,

England). Images were obtained using an acceleration voltage of 15 kV. Prior to microscopy analysis, samples were diluted in water (1:1000, v/v) dripped on stub and dried at room temperature under vacuum. Dried samples were coated with gold (92 Aº) using SC7620 Sputter Coater Polaron (VG Microtech, England).

2.4.5. Fourier transform infrared (FTIR)

Infrared spectra of nanoparticles with and without bromelain were obtained in a infrared spectrophotometer with Fourier transform (Shimadzu Scientific Instruments, Model 8300, Japan), operating at 4000 to 650 cm^{-1}, with 4 cm^{-1} resolution. Bromelain, TPP and chitosans were also characterized by FTIR technique for data comparison.

2.5. Nanoparticles stability

Nanoparticles long-term stability (until 30 days) was extrapolated by short-term measurements (this is, 33 minutes) performed by dispersion analyzer of multi-wavelength LUMiSizer® (LUM GmbH, Berlin, Germany). LUMiSizer allows accelerating separation of dispersion components by a Relative Centrifugal Force (RCF) application, varying from 5 to 2325 RCF, which accelerates materials movement in relation to gravity. Thus, samples of chitosan and chitosan-bromelain nanoparticles were subjected to this analysis (Brunelli et al., 2016).

2.6. Rheological analysis

Mechanical properties of nanoparticles suspensions were evaluated using a TA.XT Plus texturometer (Stable Micro Systems Ltda., Surrey, England) with Back Extrusion Rig platform. This platform comprises a sample container, which is centrally located below a disk plunger responsible to perform a compression test leading to product extrusion above and around disk border. Firmness (g), consistency (gs), cohesiveness (g) and viscosity index (gs) parameters were determined using the instrument software based on graph of force (g) as a function of time (seconds).

2.7. Statistical analysis

All measurements were performed in triplicate, and all results are expressed as mean ± standard deviation values. Statistical significance was established at $p < 0.05$, and were calculated using one-way analysis of variance or Student's T-test.

3. Results and Discussion

3.1. Production and characterization of nanoparticles

Nanoparticles with and without bromelain produced with different chitosan types were physically characterized by DLS, zeta potential and NTA (Figure IV.1 and Table IV.1). Results obtained show that regardless of the type of chitosan used, the addition of bromelain lead to a decrease of the nanoparticles mean diameter and zeta potential values, when compared to the respective blank nanoparticles. According to Hebbar et al. (2012), at pH 5.0 bromelain is negatively charged, which may favor electrostatic interaction with chitosan's positively charged amine groups, decreasing both size and surface charge of nanoparticles.

Except for LAC nanoparticles, bromelain addition leads to an increase of the polydispersity index. Theoretically, PDI for monodisperse nanoparticles should be zero, however PDI lower than 0.1 can also be considered monodisperse, while systems with PDI from 0.1 to 0.4 are considered moderately polydisperse (Bhattacharjee, 2016). Given this, only LMW-bromelain nanoparticles can be accepted and considered moderate polydisperse systems, while other chitosan-bromelain nanoparticles are highly polydisperse systems.

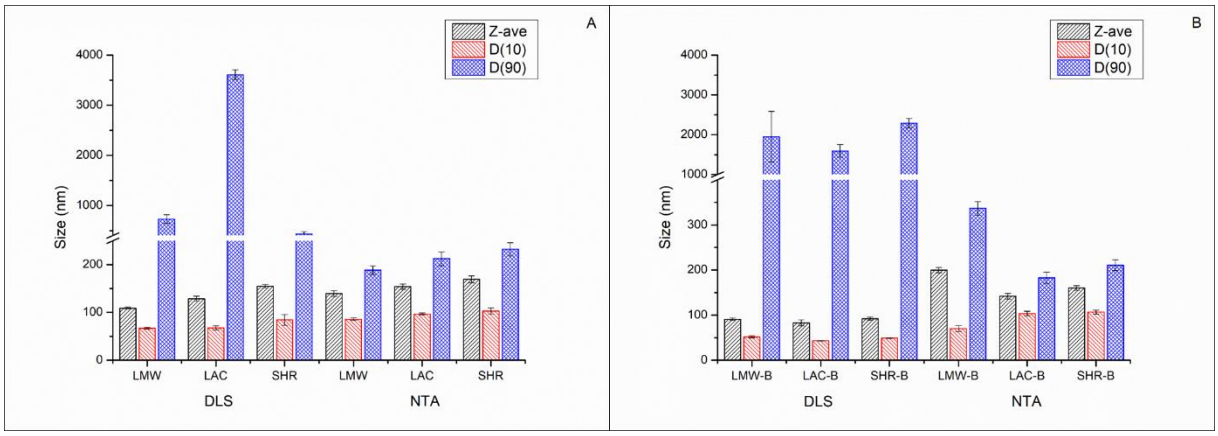

Figure IV.1. Chitosan (A) and chitosan-bromelain (B) nanoparticles size distribution. LMW = low molecular weight chitosan nanoparticles; LAC = chitosan oligosaccharide lactate nanoparticles; SHR = chitosan from shrimp shells nanoparticles; B = bromelain; Z-ave = mean diameter; D(10) = size below which 10% of material is contained; D(90) = size up to and including which 90% of material is contained.

Table IV.1. Physical characterization of chitosan and chitosan-bromelain nanoparticles.

NP	PDI	Zeta Potential (mV)	Concentration (x10^{11} particles/mL)
LMW	0.350 ± 0.051	30.6 ± 2.7	4.7 ± 0.4
LAC	0.541 ± 0.014	28.4 ± 2.4	14.3 ± 0.8
SHR	0.327 ± 0.006	33.2 ± 3.3	9.8 ± 0.2
LMW-B	0.358 ± 0.062	28.9 ± 1.7	6.9 ± 0.3
LAC-B	0.498 ± 0.034	26.3 ± 2.5	19.9 ± 1.3
SHR-B	0.427 ± 0.018	30.0 ± 2.4	1.7 ± 0.8

**Results presented as mean ± SD of three measurements. NP = nanoparticles; PDI = polydispersity index; LMW = low molecular weight chitosan nanoparticles; LAC = chitosan oligosaccharide lactate nanoparticles; SHR = chitosan from shrimp shells nanoparticles; B = bromelain.*

To determine encapsulation efficiency, nanoparticles were centrifuged in ultrafiltration devices and filtered solution was submitted to protein and enzymatic activity quantification assays (Table IV.2). LAC-B nanoparticles presented the highest protein encapsulation, while LWM-B nanoparticles presented the highest encapsulation of enzymatic activity.

Table IV.2. Total proteins concentration, enzymatic activity and encapsulation efficiency of bromelain.

	Protein		Enzymatic Activity	
	Amount (mg)	EE (%)	Activity (U)	EE (%)
Bromelain Solution	1.68 ± 0.46	-	20.4 ± 0.1	-
LMW-B	0.18 ± 0.03	89.1	1.6 ± 0.5	91.9
LAC-B	0.04 ± 0.01	97.7	2.8 ± 0.1	86.3
SHR-B	0.27 ± 0.04	84.1	4.1 ± 1.3	79.8

**Results presented as mean ± SD of three measurements. LMW = low molecular weight chitosan nanoparticles; LAC = chitosan oligosaccharide lactate nanoparticles; SHR = chitosan from shrimp shells nanoparticles; B = bromelain.*

Scanning electron microscopy (SEM) was used to evaluate nanoparticles morphology. In SEM images (Figure IV.2), it was possible to confirm the spherical shape with smooth and regular surface of LMW (Figure IV.2A), LMW-B (Figure IV.2B), LAC (Figure IV.2C), LAC-B (Figure IV.2D) and SHR (Figure IV.2E) nanoparticles. SHR-B nanoparticles (Figure IV.2F),

however, present non-regular and non-uniform shape and surface. SHR-B image also show a diffuse network that may be non-encapsulated bromelain, once SHR-B nanoparticles presented the lowest encapsulation efficiency in terms of protein and enzymatic activity.

In SEM images, all nanoparticles showed about 1 μm of diameter, bigger than size obtained by DLS and NTA measurements. Even soft drying process, as conducted at room temperature, can lead to nanoparticles aggregation leading to microparticles formation (Rampino et al., 2013). Chitosan has been studied for hydrogel nanoparticles production through various methods, including ionotropic gelation with TPP. These hydrogel-nanoparticulated materials have hydrogel and nanoparticle characteristics simultaneously. Therefore, chitosan nanoparticles present hydrophilicity, flexibility and high water absorption similar to hydrogels (Hamidi et al., 2008). Thus, bigger particles observed on SEM images can also be attributed to water absorption by particles that were not completely dry.

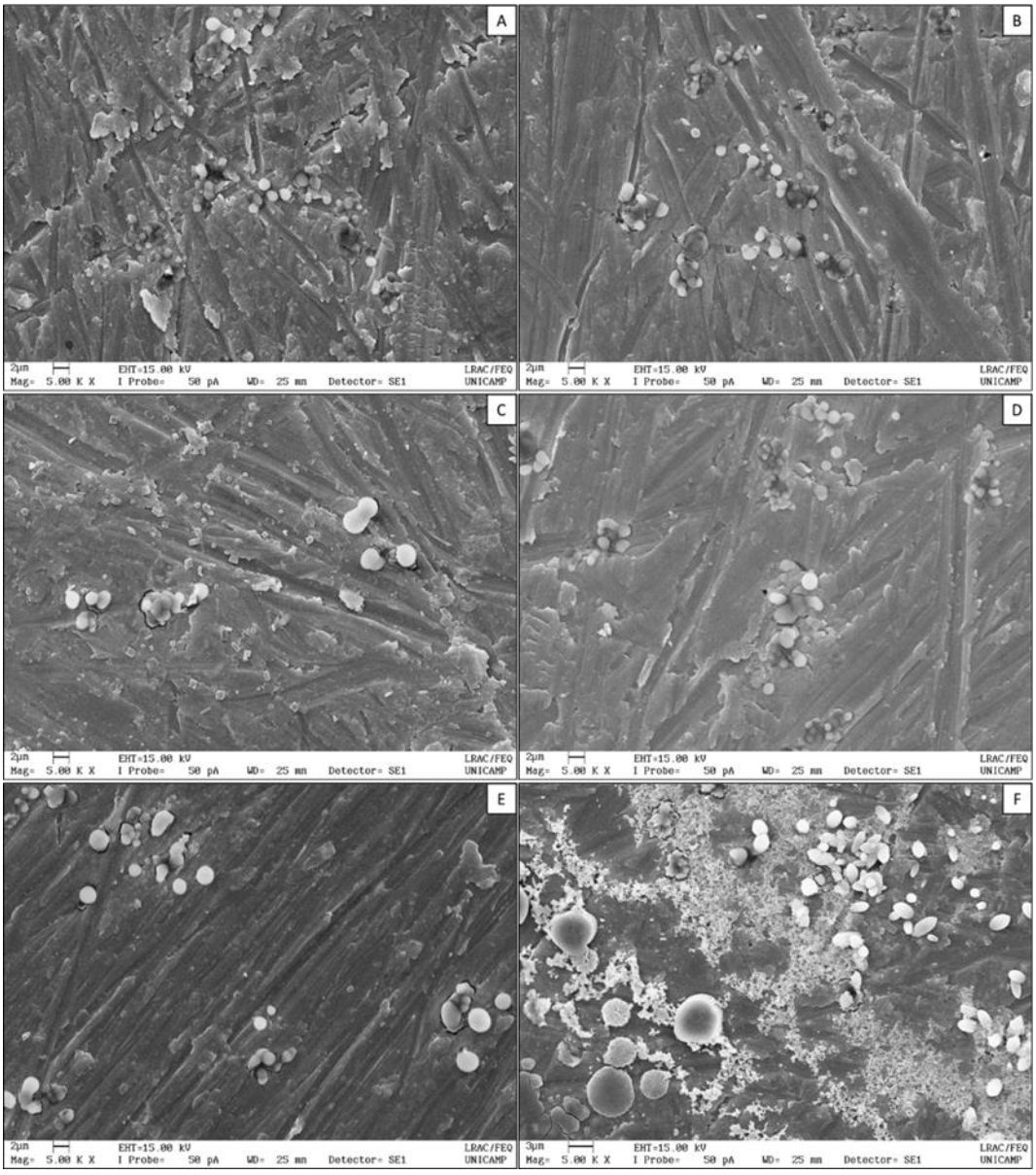

Figure IV.2. Scanning electron microscopy images (SEM) of LMW (A), LMW-B (B), LAC (C), LAC-B (D), SHR (E) and SHR-B (F) nanoparticles. LMW = low molecular weight chitosan nanoparticles; LAC = chitosan oligosaccharide lactate nanoparticles; SHR = chitosan from shrimp shells nanoparticles; B = bromelain.

FTIR spectra of different types of chitosan (Figure IV.3A) show characteristic polysaccharides peaks in fingerprint region from 1156 to 890 cm^{-1} (Pereira et al., 2015; Santos et al., 2003), as peaks correspondent to C-H on rings, C-O of alcohols, and C-O-C asymmetric characteristic of glycoside bonds. In chitosans' spectra is also possible to observe peaks around 3300 cm^{-1}, corresponding to hydrogen bonds of O-H groups, which overlaps N-H stretch band (Pereira et al., 2015).

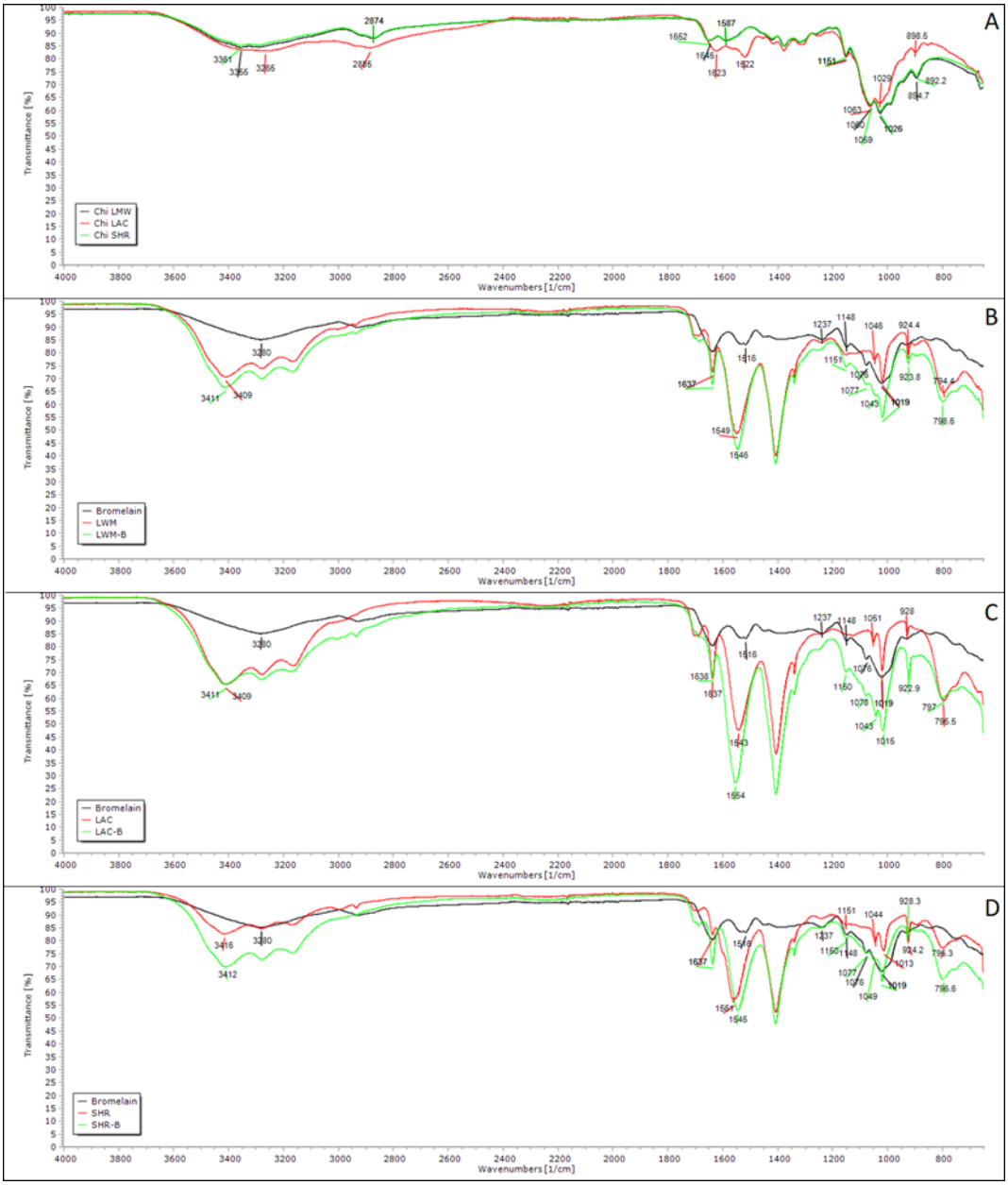

Figure IV.3. Fourier transform infrared spectra of different types of chitosan (A) and nanoparticles prepared with low molecular weight (B), oligosaccharide lactate (C) and from shrimp shells (D) chitosan. LMW = low molecular weight chitosan nanoparticles; LAC = chitosan oligosaccharide lactate nanoparticles; SHR = chitosan from shrimp shells nanoparticles; B = bromelain.

Peaks corresponding to C=O and N-H bonds are present in all samples, however with displacements: for LMW chitosan they appear at 1645 and 1587 cm^{-1}, for LAC chitosan at 1622 and 1521 cm^{-1}, and for SHR chitosan at 1651 and 1587 cm^{-1}, respectively. According to He et al. (2016), lower intensity of C=O peaks are associated to higher deacetylation degree. LMW and SHR chitosans presented bands with similar intensity, which is an indicative of same deacetylation degree. LAC chitosan showed a higher intensity band, even being reported higher

deacetylation grade (> 90%), which may be explained for the presence of C=O in lactate (Cervera et al., 2011).

Bromelain exhibits characteristic enzymatic peptide bond peak at 3280 cm^{-1}, and also peaks at 1634 cm^{-1} and 1516 cm^{-1} represents C=O and N-H groups, confirming amino acids presence. The peak at 1236 cm^{-1} can be attributed to C-N bond on aliphatic amine (Ataide, Cefali, et al., 2017; Ataide, de Carvalho, et al., 2017; Devakate et al., 2009; Soares et al., 2012).

Nanoparticles composed by different chitosan, with and without bromelain, were also analysed by FTIR. All nanoparticles presented absorption band around 3400 cm^{-1} equivalent to hydrogen interaction and O-H vibration, with intensity increase when compared to the same peak in different types of chitosan, which suggests an increase in hydrogen bonds in nanoparticles without bromelain (Wu et al., 2005). This same band presents even higher intensity in bromelain nanoparticles. All nanoparticles spectra present a peak around 795 cm^{-1}, which may be attributed to vibrations related to P–O and P–O–P bonds (Antoniou et al., 2015; Knaul et al., 1999).

In the blank nanoparticles is possible to observe displacement of C=O and N-H peaks, when compared to chitosans' peaks. These displacements indicates interaction between chitosan's amino groups with TPP's phosphate groups (Antoniou et al., 2015; Knaul et al., 1999). Comparing this very same peak region, it is possible to note an intensity increase in both peaks in chitosan-bromelain nanoparticles, which can be attributed to bromelain incorporation once bromelain also absorbs in the same frequency (Devakate et al., 2009; Soares et al., 2012). Chitosan-bromelain nanoparticles also present peaks around 1148 and 1076 cm^{-1} similar to bromelain spectrum, which were not observed in chitosan nanoparticles.

3.2. Nanoparticles stability

LumiSizer device detects light transmitted through the sample in space and time solved by an optoelectronic sensor (Ullmann et al., 2017). Being that, the samples must have a sufficiently high turbidity, which depends on the particle size and shape, as well as its optical properties (Babick, 2016). Therefore, samples must have sufficient high turbidity, which depends on the particle size and shape, as well as its optical properties (Babick, 2016). Previous studies showed that initial normalized intensity between 10 and 30% provides maximum reliability, but any transmission value in the range of 5% to 80% is typically acceptable (Babick, 2016; Ullmann et al., 2017). All nanoparticles showed transmittance profile higher than 90% during the tested period (data not shown), indicating that samples are not suitable for this technique or it is much diluted.

3.3. Rheological analysis

Viscoelastic properties of nanoparticles suspension was evaluated trough back extrusion test. In this test, maximum force applied is taken as firmness measurement, and grater values indicate a denser sample consistency. The area under the curve up to this point is taken as a consistency indication, which refers to "firmness", "thickness" or "viscosity" of a liquid or a semi-solid. This test also provides an indication of viscosity, which is the result of sample weight lifted mainly in disc upper surface on its return, that is, a measure of resistance to disk flow (Cevoli et al., 2013; Pawar & Pande, 2015).

Table IV.3. Viscoelastic properties of nanoparticles suspensions, obtained by back extrusion rig test.

NP	Firmness (g)	Consistency (gs)	Cohesiveness (g)	Viscosity Index (gs)
LMW	980.6 ± 51.9	1629.8 ± 346.6	-909.4 ± 32.0	393.3 ± 386.5
LMW-B	915.9 ± 25.5	1626.4 ± 35.7	-860.8 ± 117.7	412.3 ± 29.3
LAC	935.3 ± 16.5	1695.1 ± 36.3	-906.2 ± 88.4	546.9 ± 108.5
LAC-B	925.6 ± 32.0	1685.1 ± 240.7	-809.1 ± 69.3	637.0 ± 142.7
SHR	873.8 ± 21.0	1444.5 ± 34.4	-851.1 ± 16.5	341.0 ± 41.7
SHR-B	909.4 ± 18.3	1375.4 ± 122.5	-802.6 ± 35.8	266.4 ± 131.2

** Results presented as mean ± SD of three measurements. g = grams; s = seconds; LMW = low molecular weight chitosan nanoparticles; LAC = chitosan oligosaccharide lactate nanoparticles; SHR = chitosan from shrimp shells nanoparticles; B = bromelain.*

Cohesion is determined by intermolecular attraction, which maintain together the elements of a body or mass of material. It is related to product internal viscosity and is usually determined by measuring the force required to remove an item from product. In this test, maximum negative force is taken as an indication of the sample's cohesiveness (Cevoli et al., 2013; Pawar & Pande, 2015).

Nanoparticles made with SHR chitosan presented the lowest viscosity index between all used chitosan types, and this value decreased with bromelain incorporation (Table IV.3). However, for other chitosan types, viscosity index increased with bromelain addition. The biggest cohesiveness was found for LMW chitosan nanoparticles without bromelain, and this

cohesiveness decreased with bromelain addition. The same behavior was found for other chitosan types, indicating that bromelain addition makes the suspension less cohesive.

4. Conclusions

Our results confirm that all tested chitosan types (LMW, LAC and SHR) were suitable for the production of nanoparticles, as demonstrated by dynamic light scattering (DLS) and Fourier transform infrared (FTIR) spectroscopy. For all chitosan types, particle size and zeta potential decreased and polydispersity index increased after bromelain addition. Bromelain has been successfully loaded within chitosan nanoparticles (with encapsulation efficiency higher than 84% for proteins, and higher than 79% for enzymatic activity), and the efficiency in terms of enzymatic activity was higher for nanoparticles made with LMW chitosan. Bromelain encapsulation in chitosan nanoparticles was also confirmed by FTIR. In general, bromelain addition decreased the cohesiveness of nanoparticles suspension. It was not possible to obtain conclusive results in LumiSizer tests, due to samples' high transmittance. Based on obtained results, LMW-chitosan was able to encapsulate bromelain, generating moderate polydisperse systems with spherical shape, with high encapsulation efficiency (89.1% for proteins and 91.9% for enzymatic activity).

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CAPÍTULO V. "FREEZE-DRIED CHITOSAN NANOPARTICLES TO STABILIZE AND DELIVER BROMELAIN"

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Abstract

Bromelain has many uses for its properties including being a wound healing and circulatory improvement agent. However, bromelain is usually unstable under stress conditions, which results in a decrease of enzymatic activity and limits its applications. Encapsulation of bromelain in nanoparticles can increase its stability, efficacy and safety, besides modification of its release kinetics. The natural polymer chitosan forms nanostructures that can trap the enzyme, maintaining the claims of biocompatibility, biodegradability and natural source of the active and the formulation. Considering the above, chitosan-bromelain nanoparticles were produced by ionic crosslinking and resulted in spherical particles (scanning electron microscopy) of 100.9 ± 0.5 nm and polydispersity index of 0.222 ± 0.012 (mean average, dynamic light scattering). Encapsulation efficiency was 87.4% of total protein concentration, corresponding to 80.7% of enzymatic activity, a high and desirable rate. However chitosan-bromelain did not present desired stability when stored in aqueous suspension and formulations were freeze-dried. Glycine or maltose were used as potential lyoprotectors; resultant products presented elegant cakes with short resuspension time, slightly altering nanoparticles size and increasing encapsulation rate of the previous liquid form. Therefore, freeze-dried chitosan-bromelain nanoparticles can effectively improve bromelain stability when compared to liquid forms, which allow further *in vivo* applications as a dried powder for oral/topical administration or as a raw material for other dosage forms.

Keywords: bromelain; nanoencapsulation; formulation stability; freeze-drying; technology platforms to enhance stability

1. Introduction

Bromelain is a collective name for proteolytic enzymes or proteases found in pineapple tissues (*Ananas comosus* L.) and several species of the Bromeliaceae family (Hennrich et al., 1969; Taussig and Batkin, 1988; Doko et al., 1991; Maurer, 2001; Fitzhugh et al., 2008; Chobotova et al., 2010; Chaurasiya and Umesh Hebbar, 2013). Due to its proteolytic activity, bromelain has potential applications in the cosmetic, pharmaceutical and food industries. From the Seligman study that showed its action as an anti-inflammatory agent in 1962, several studies support the use of bromelain extracts in different conditions (Seligman, 1962; Taussig and Batkin, 1988; Salas et al., 2008; Chobotova et al., 2010; Amid et al., 2011; Ferreira et al., 2011). Some studies, in particular, have demonstrated the potential use of bromelain in healing processes. Maurer (2001) demonstrated that this enzyme has benefits for wound healing, specifically reducing edema, bruising and pain. In burns, bromelain acts by hydrolyzing the devitalized tissue, both *in vivo* and *in vitro*, which increases the healing capacity.

Although bromelain desirable properties, its stability in topical formulations is an issue, even when stored under low temperatures (Pereira et al., 2014; Spir et al., 2015; Lourenço et al., 2016; Ataide et al., 2017). It is known that bromelain cleaves, preferably, glycyl, alanyl and leucyl peptide bonds, and its catalytic mechanism involves the oxidation of sulfhydryl groups (-SH), resulting in the formation of disulfide bonds (Maurer, 2001). This process leads to autoproteolytic cleavage, thereby reducing bromelain enzymatic activity (Bala et al., 2012). Pereira et al. (2014) demonstrated that formulations with bromelain, when stored at 37 °C, lost almost all their enzymatic activity. They attributed this fact to the autodegradation (autolysis or autodigestion) of bromelain, since 37 °C has been considered the optimal temperature for its proteolytic activity (Pereira et al., 2014).

In recent years, the development of nanoscale materials and devices has attracted great interest, and successful examples can already be found in therapy (Pachioni-Vasconcelos et al., 2016; Kumar et al., 2018; Kuo and Rajesh, 2018). Nanostructures as drug delivery systems are key to overcome challenges associated with drug therapy, including poor solubility, poor permeability, short half-life in the target organism and high toxicity (Kammona and Kiparissides, 2012; Bernkop-Schnürch, 2013). Due to its characteristics of adhesiveness, biocompatibility, biodegradability and low toxicity, chitosan has become a potentially attractive material for several uses, mainly in the pharmaceutical area (Sogias et al., 2008). This polysaccharide has been used as a polymer system in the modified release of drugs of various therapeutic classes, such as antibiotics, anti-inflammatories, antihypertensives, peptides and proteins (Bernkop-Schnürch, 2000; Florea et al., 2006; Boonyo et al., 2007; Pachioni-

Vasconcelos et al., 2016). In addition, the use of chitosan for the treatment of wounds and burns has been studied, based on its hemostatic capacity and its accelerating effect on wound repair with better final static effect. The ease of adhesion of chitosan, as well as its antifungal, bacteriostatic and oxygen permeability properties are very attractive properties for topical use (Argüelles, 2004; Jayakumar et al., 2011; Malmiri et al., 2012).

When stored in liquid form, these systems may present physical instabilities (particles aggregation or fusion) and/or chemical instabilities (hydrolysis of polymer materials or drug leakage), limiting nanoparticles applications (Abdelwahed et al., 2006). Besides physical and chemical instabilities, nanoparticles suspensions are susceptible to microorganisms development and growth (Fonte et al., 2016). A promising approach to remove water and ensure long-term stability of nanoparticulated systems is freeze-drying (Sylvester et al., 2018), which consists in water removal from a frozen sample under vacuum by sublimation and desorption (Abdelwahed et al., 2006). Freeze-drying process, also known as lyophilization, comprises three main steps namely freezing, primary drying and secondary drying. Even though it is considered a mild process for concentrating or drying biologically active substances, this process generates various stresses to samples during freezing and drying steps. This stability issue for proteins and nanoparticles can be mitigated by the addition of excipients (cryo- and lyoprotectants) to the formulation (Arakawa et al., 2001; Roy and Gupta, 2004; Fonte et al., 2016).

In this scenario, bromelain encapsulation into chitosan nanoparticles is intended to increase its stability, efficacy and safety, besides modification of its release kinetics. Freeze-drying process of chitosan-bromelain nanoparticles was also studied, aiming the development of an innovative and stable nanoscale delivery system, increasing its therapeutic use.

2. Materials and Methods

2.1. Materials

Low molecular weight chitosan (75-85% deacetylated), bromelain (≥ 3 units/mg protein, ≥30% protein biuret), azocasein and Bradford reagent were purchased from Sigma-Aldrich (São Paulo, Brazil). All other reagents were purchased at analytical grade.

2.2. Screening of chitosan nanoparticles formulation

Chitosan nanoparticles were prepared according to the ionic gelation method (Servat-Medina et al., 2015; Goycoolea et al., 2016). Briefly, chitosan solutions were prepared by dissolving chitosan into 1% (v/v) acetic acid solution, and pH was corrected to 5.0 by adding

NaOH solution, while sodium tripolyphosphate (TPP) were dissolved into distilled water. Chitosan and TPP solutions were filtered in membranes of 0.45 and 0.22 µm, respectively. Nanoparticles were formed by adding a determined volume of TPP solution dropwise onto a determined volume of chitosan solution under magnetic stirring (Fisatom, Mod 753E, São Paulo, Brazil). For an initial formulation screening, a factorial design 22 with triplicate of central point was used. The mass relation TPP/Chitosan (10, 20 and 30%, w/w) and stirring velocity (350, 550, and 750 rpm) were used as input, while average particle size, polydispersity index (PDI) and zeta potential by dynamic light scattering (DLS) were used as output. All experiments were analyzed using Minitab version 17 (Minitab Inc., USA). Aiming to compare those particles with chitosan-bromelain nanoparticles, 1 mL of 0.22 µm-filtered water was added after TPP addition.

2.3. Bromelain solution

Bromelain solution was prepared by dissolving bromelain in distilled water (10 mg/mL) and filtering it with 0.22 µm membranes. Total protein concentration and enzymatic activity were determined in this solution. Protein concentration was determined according with Bradford method (Bradford, 1976), reading the absorbance at 595 nm (Multiscan GO, Thermo Scientific, Sweden). Enzymatic activity was determined using the azocasein method (Sarath et al., 1989; Coelho et al., 2016). Bromelain was allowed to cleave the substrate, azocasein, at 37 °C for 10 minutes; trichloroacetic acid was then added to precipitate non-hydrolyzed azocasein and stop the reaction. Cleavage of azocasein caused the release of tyrosine residues, which were detected at 440 nm by spectroscopy (Genesys 10S UV-Vis, Thermo Scientific, Sweden). Enzymatic activity was calculated in activity units (U/mL) and was defined as the amount of bromelain needed to produce 1 mmol of tyrosine per minute at 37 °C.

2.4. Chitosan-bromelain nanoparticles

According to factorial design, the nanoparticle with better diameter, PDI and zeta potential was used to incorporate bromelain. For chitosan-bromelain production, 1 mL of bromelain solution was added after TPP dripping. Simultaneously, chitosan nanoparticles were produced as a control, and for this bromelain solution was replaced by the same volume of distilled and filtered water.

2.5. Bromelain encapsulation efficiency

To determine bromelain encapsulation efficiency, nanoparticle suspensions were centrifuged (Centrifuge 5810R, Eppendorf, Germany) for 10 minutes at 14,000g using 0.5 ml ultrafiltration devices with 100 kDa membrane (Amicon® Ultra 100k, Millipore, Germany). Encapsulation efficiency was determined by the difference in protein concentration and enzymatic activity in the initial bromelain solution and in the filtered solution, according to equation I, where $Brom_i$ is the initial bromelain added and $Brom_n$ is the nonencapsulated bromelain.

$$\%EE = \frac{Brom_i - Brom_n}{Brom_i} \times 100 \qquad \text{Equation I}$$

2.6. Nanoparticles characterization

2.6.1. Dynamic light scattering (DLS) and zeta potential

Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipment was used to determine average particle size, particle size distribution, polydispersity index, and zeta potential of chitosan and chitosan-bromelain nanoparticles. Average particle size and polydispersity index were determined by dynamic light scattering, where the intensity of light scattered was used to calculate the mean hydrodynamic diameter (Z-average mean), based on the Stokes–Einstein equation, which assumes that the particle is spherical. Zeta potential analysis was carried out using laser doppler microelectrophoresis at 25 °C, and potentials were automatically calculated from the electrophoretic mobility using the Smoluchowski's approximation (Servat-Medina et al., 2015; Oliveira et al., 2017).

2.6.2. Nanoparticles tracking analysis (NTA)

Nanoparticles mean diameter and size distribution were also determined by nanoparticles tracking analysis (NTA) using the NanoSight NS300 (Malvern, United Kingdom) equipment. This analysis also allow the determination of concentration of nanoparticles per volume. For this, nanoparticles suspension was diluted 1000 times in Milli-Q water.

2.6.3. Scanning electron microscopy (SEM)

Nanoparticles morphological characteristics were observed using a Leo 440i scanning electron microscopy with 6070 X-ray dispersive energy detector (LEO Electron Microscopy, England). SEM images were obtained using an accelerating voltage of 15 kV. Before analysis,

samples were lyophilized for 24 hours and coated with gold (92 Aº) using SC7620 Sputter Coater Polaron (VG Microtech, England).

2.7. Bromelain release in vitro

*In vitro b*romelain release from nanoparticles was studied in saline phosphate buffer (PBS) pH 7.4 and water. Briefly, one part of chitosan-bromelain nanoparticle suspension was mixed with four parts of release medium and kept at 37 °C with constant magnetic stirring. At pre-determined time intervals, samples were collected and centrifuged for 10 minutes at 14,000g using 0.5 mL ultrafiltration devices with 100 kDa membrane. Protein concentration was determined in filtered solutions using micro-Bradford performed according with Sigma-Aldrich technical bulletin.

2.8. Nanoparticles stability studies

For stability studies, chitosan and chitosan-bromelain nanoparticles suspension were evaluated according with stability testes recommended at "Stability studies guideline" by Brazilian`s National Health Surveillance Agency (ANVISA) (Anvisa, 2005). Samples were stored in glass flasks at tree conditions: (i) room temperature (25 ± 2 ℃) exposed to light, (ii) room temperature (25 ± 2 ℃) protected from light, and (iii) refrigerator (5 ± 2 ℃). Samples were assayed daily during 15 days for preliminary stability study and on pre-determined days (0, 7, 15, 30, 45, 60, 90) for accelerated stability studies.

During preliminary stability, samples were submitted to general evaluations, such as macroscopic characteristics (color, odor and appearance) and pH evaluation. For accelerated stability, in addition to general evaluations, samples were submitted to specific bromelain and nanoparticles parameters, such as total protein concentration and enzymatic activity determination, and nanoparticles characterization by zeta potential and dynamic light scattering for particle size and polydispersity index determination.

2.9. Freeze-drying

2.9.1. Lyoprotectants evaluation and collapse temperatures

Maltose, trehalose and glycine were tested as possible diluents and lyoprotectants for nanoparticles freeze-drying process. Collapse temperatures of chitosan-bromelain nanoparticles without and with 3% (w/v) lyoprotectants were determined by a microscope coupled to a lyophilization module, Lyostat 2, model FDCS 196 (Linkam Instruments, Surrey,

UK), equipped with liquid nitrogen freezing system (LNP94 / 2) and controller of programmable temperature (TMS94, Linkam). Pressure was monitored through a Pirani valve. The equipment was calibrated with aqueous solution of NaCl (eutectic temperature of -21.1 °C). Direct observation of freezing and freeze drying was done by a Nikon polarized light microscope, model Elipse E600 (Nikon, Japan), and in heating-cooling ramps of 5 ° C/min. Data were analyzed by the Linksys 32 software.

2.9.2. Freeze-drying process

For an initial process study, a factorial design 2^3 with duplicate of each point was used, using as inputs the lyoprotector, its concentration, and lyophilization process. Samples were submitted to fast freezing with liquid nitrogen, and lyophilized in Lyostar 3 Freeze-Drier (SP Scientific, USA). Primary drying was carried rising 5 °C in temperature from -45 °C to 10 °C, waiting for vials temperature to reach shelfs temperature. After dried, samples were reconstituted in the same initial volume in distilled water, and were physic-chemically characterized by dynamic light scattering and encapsulation efficiency, which were used as outputs.

Freeze-dried products was submitted to thermogravimetric analysis (TGA-50M, Shimadzu, Japan) in order to determine their residual moisture. Accurately weighted dried nanoparticles samples (around 10 mg) were heated at 10 °C/min from 25 up to 300 °C, under nitrogen atmosphere at a flow rate of 50 mL/min. Residual moisture was determined by stable weight-loss (%) at temperature around 100 °C (Sylvester et al., 2018).

2.10. Statistical analysis

All measurements were performed in triplicate. All results are expressed as mean ± standard deviation values, and were calculated using one-way analysis of variance or Student's T-test. Statistical significance was established at $p < 0.05$.

3. Results and Discussion

3.1. Chitosan nanoparticles formulation

A factorial design 2^2 with triplicate of central point was used to optimize the nanoparticles formulation (Table V.1). During this formulation screening, 1 mL of 0.22 µm-filtered water was added after TPP, so nanoparticles could be further compared.

Table V.1. Factorial design 2^2 independent variables and chitosan nanoparticles mean size, polydispersity index and zeta potential.

NP	Input		Output		
	TPP/Chi (m/m)	Agitation (rpm)	Diameter (nm)	PDI	Zeta Potential (mV)
1	10%	350	135.1 ± 4.0	0.261 ± 0.015	27.3 ± 3.0
2	30%	350	124.6 ± 0.1	0.178 ± 0.016	23.0 ± 1.7
3	10%	750	127.6 ± 1.8	0.264 ± 0.029	26.4 ± 3.7
4	30%	750	114.6 ± 1.9	0.217 ± 0.031	23.5 ± 2.0
5	20%	550	150.6 ± 5.0	0.302 ± 0.049	28.3 ± 1.2
6	20%	550	146.9 ± 2.7	0.211 ± 0.009	23.3 ± 1.7
7	20%	550	140.3 ± 3.8	0.212 ± 0.028	22.3 ± 1.3

**Results presented as mean ± SD of three measurements. 1mL of 0.22µm-filtered water was added to each formulation after TPP addition, so blank nanoparticles could be further compared with nanoparticles with bromelain. TPP = sodium tripolyphosphate; Chi = chitosan; PDI = polydispersity index.*

For nanoparticle optimization, TPP/Chitosan mass relation of 10, 20 and 30% (w/w) and magnetic stirring at 350, 550 and 750 were chosen based on different studies conducted with chitosan (Calvo et al., 1997; Gan et al., 2005; Tang et al., 2007; Servat-Medina et al., 2015). Resulting nanoparticles were characterized by DLS, which is one of the most frequently used methods to obtain an average diameter of nanoparticles dispersed in liquids providing reasonably accurate results (Moreno-Martin et al., 2017). Ouputs were statistically analysed using pareto diagrams (Figure V.1), and any parameter interfere significantly in particle size, polydispersity index or zeta potential. Thus, nanoparticle 2, which was composed for 30% TPP/chitosan (w/w) and agitation of 350 rpm, was chosen to continue the study and incorporate bromelain, once it presented the lowest PDI.

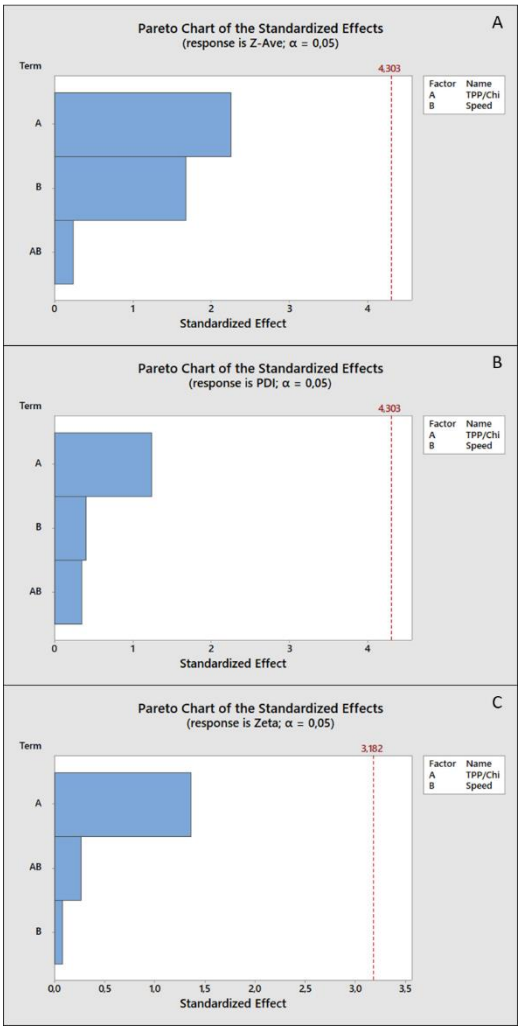

Figure V.1. Pareto diagrams of the factorial design relating the studied variables to mean size in DLS (A), polydispersity (B), and zeta potential (C).

3.2. Chitosan-bromelain nanoparticles production

Chitosan-bromelain nanoparticles were produced using the chosen parameters, with the addition of 1 mL of a bromelain solution (10 mg/mL) right after TPP solution, and nanoparticles were characterized for size determination using ZetaSizer and NanoSight. DLS is the most frequently used technique to measure nanoparticles size distribution and it provides reasonably accurate results for strictly monodisperse nanoparticles. However, DLS is unable to distinguish between nanoparticles with slight differences in diameter or to precisely resolve polydisperse samples (Moreno-Martin et al., 2017). By DLS measurement chitosan-bromelain nanoparticles have shown a smaller size than chitosan nanoparticles, and a reduction in zeta potential (Table V.2). According with Hebbar et al. (2012), bromelain is negatively charged at pH 5.0, which may have favored the electrostatic interaction with positively charged amine groups of chitosan, reducing both nanoparticles size and surface charge.

Table V.2. Physic-chemical characterization of chitosan-bromelain nanoparticles.

		DLS	NTA
Size (nm)	**Z-ave**	100.9 ± 0.5	177.2 ± 4.8
	D_{10}	56.1 ± 0.5	101.1 ± 1.3
	D_{50}	112.0 ± 2.5	143.0 ± 5.5
	D_{90}	235.0 ± 2.5	273.5 ± 19.7
PDI		0.222 ± 0.012	-
Zeta Potential (mV)		+21.9 ± 0.5	-
Concentration (particles/mL)		-	(1.25 ± 0.03) x 10^{12}

**Results presented as mean ± SD of three measurements. DLS = dynamic light scattering; NTA = nanoparticles tracking analysis; PDI = polydispersity index.*

On the other hand, NTA combines single particle and ensemble approach and it is based on the ability to detect individual particles using optical methods via scattered laser light. Size distribution is provide by the analysis of the tracked movement using the Stokes–Einstein equation. This technique also enable counting nanoparticles directly in solution, and so it is possible to determine the present number of particles (Moreno-Martin et al., 2017).

Both DLS and NTA techniques measure nanoparticles hydrodynamic diameter (Bell et al., 2012). However, they use different ways for this determination: DLS detects hydrodynamic by correlating the fluctuation in scattered light intensity over the time, while NTA records nanoparticles mobility from scattered light captured as videos, leading to differences in obtained sizes (Bhattacharjee, 2016). For chitosan nanoparticles, average size determined by DLS was higher than size by NTA, which can be attributed to DLS intrinsic propensity to detect larger particles, since the ability of a particle to scatter light is proportional to its diameter to the sixth power (Boyd et al., 2011).

In its turn, mean chitosan-bromelain nanoparticles showed an opposite behavior: size determined by DLS was smaller than size by NTA. DLS has low resolution, being unable, for example, to distinguish between particles of 90 and 110 nm, which will appear as a broad peak with high PDI (Kaszuba et al., 2007; Bhattacharjee, 2016). NTA also has its drawbacks. Smaller and faster-moving nanoparticles can not be tracked for as long as the larger ones, having a greater uncertainty associated with individual diameters (Bell et al., 2012).

For encapsulation efficiency determination, chitosan nanoparticles were also submitted to the same procedure as chitosan-bromelain nanoparticles, and used as control. Chitosan nanoparticles did not present a significant protein concentration and neither enzymatic activity, and thus it was possible to conclude that the suspension did not interfere in those analyses. The initial bromelain solution presented 2.0 ± 0.3 mg/mL of total protein and 23.3 ± 1.6 U/mL. Protein concentration and enzymatic activity were also determined in the resulting filtered solution (0.25 ± 0.01 mg/mL and 4.5 ± 0.6 U/mL, respectively) for encapsulation efficiency calculation, which was found to be 87.0 ± 5.1% of total protein concentration, corresponding to 80.7 ± 1.1 % of enzymatic activity of bromelain.

3.3. Nanoparticles morphology

Nanoparticles morphology was evaluated using Scanning Electron Microscopy. SEM images obtained at 15 kX showed chitosan (Figure V.2A) and chitosan-bromelain (Figure V.2B) nanoparticles had spherical shape, smooth surface, and size diameter approximately 1 µm, which was larger than size obtained by DLS and NTA measurements. Even mild drying process, such as room temperature, may lead to nanoparticles aggregation, leading to microparticles formation (Rampino et al., 2013).

Chitosan has been studied to produce hydrogel nanoparticles via different methods, including ionotropic gelation with TPP. These hydrogel nanoparticulate materials hold, at the same time, features and characteristics from both hydrogels and nanoparticles. Therefore, chitosan nanoparticles may also present the hydrophilicity, flexibility, and high water absorptivity from hydrogels (Hamidi et al., 2008). Thus, larger particles observed in SEM images could also be attributed to water absorption by particles, which did not completely dried.

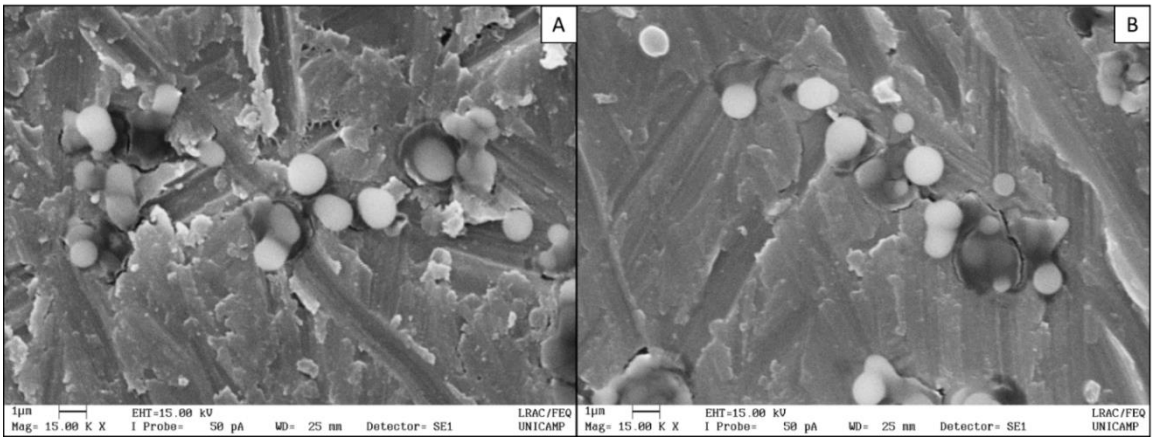

Figure V.2. Scanning electron microscopy of chitosan nanoparticles (A), chitosan-bromelain nanoparticles (B).

3.4. Bromelain release in vitro

The *in vitro* release profile of bromelain from chitosan-bromelain nanoparticles was evaluated for 48 hours in PBS pH 7.4 and water (Figure V.3). An initial burst release of 42.2 ± 21.1% is observed in the first 2 hours in water, while a release of 31.4 ± 0.2% is observed after 6 hours in PBS. Thus, it is possible to suggest that in biological buffer, bromelain showed a slower release when compared with water. After 2 and 6 hours in water and buffer, respectively, is possible to note a decrease in total protein released amount. This decrease was not observed for authors incorporating bromelain in PLGA nanoparticles (Bhatnagar et al., 2015; Bhatnagar et al., 2016), and maybe explained by procedure limitations such as bromelain could be aggregating in release medium, not being able to pass through ultracentrifuge device membrane. Another limitation was assay used to determine protein concentration. The calibration curve of micro-Bradford assay was determined using bovine serum albumin, presenting R^2 of 0.9911, detection limit of 3.47 µg/mL and quantification limit of 10.52 µg/mL. Thus, release percentages lower than 15% were not precisely determined.

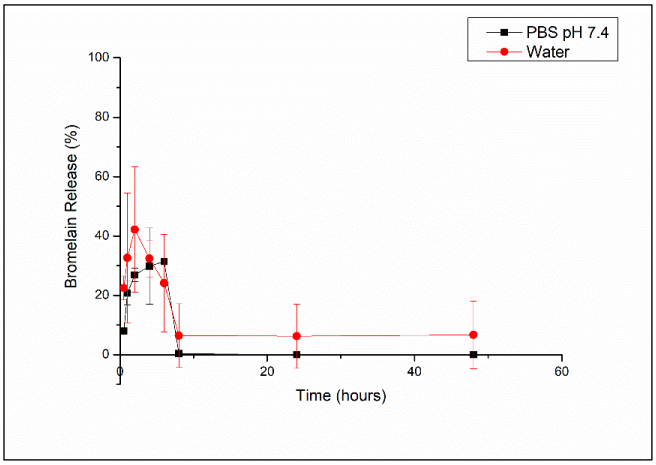

Figure V.3. In vitro *release profile of bromelain from chitosan-bromelain nanoparticles in PBS pH 7.4 and water.*

3.5. Nanoparticles stability study

For stability studies a new batch of chitosan and chitosan-bromelain nanoparticles were produced following the same procedures, using a volume 20 times greater. Right after produced, chitosan and chitosan-bromelain nanoparticles had a mean diameter of 208.7 ± 4.2 and 110.5 ±1.4 nm; PDI of 0.306 ± 0.053 and 0.302 ± 0.034; and zeta potential of 30.8 ± 1.0 and 20.3 ±

1.8 mV, respectively. This size reduction could be attributed to the use of greater volume, without changing other parameters such as time and agitation velocity.

During preliminary stability study, general parameters, such as pH value and macroscopic characteristics were evaluated. Chitosan and chitosan-bromelain nanoparticles suspension did not show any macroscopic and pH alteration. Thus, samples continued to accelerated stability study. After 7 days of study (Figure V.4), chitosan-bromelain nanoparticles presented a significant increase ($p < 0.05$, Student T-test) in mean size diameter in all studied conditions, accompanied with also significant increase in PDI and decrease in zeta potential. Increase in particle size is regularly attributed to particle agglomeration, which may be induced by adsorption of active molecules on the nanoparticles (Magenheim and Benita, 1991; Abdelwahed et al., 2006).

On the other hand, chitosan nanoparticles showed a size reduction which was significant ($p < 0.05$, Student T-test) after 15 days in room temperature exposed to light, an after 30 days in room temperature protected from light and in refrigerator (5 °C). Nanoparticles initial sizes have been reported to affect nanoparticles storage stability. According with Tsai et al. (2011), nanoparticles smaller than 120 nm, had their size significantly increased with storage time, while nanoparticles larger than 140 nm, suffered the opposite effect.

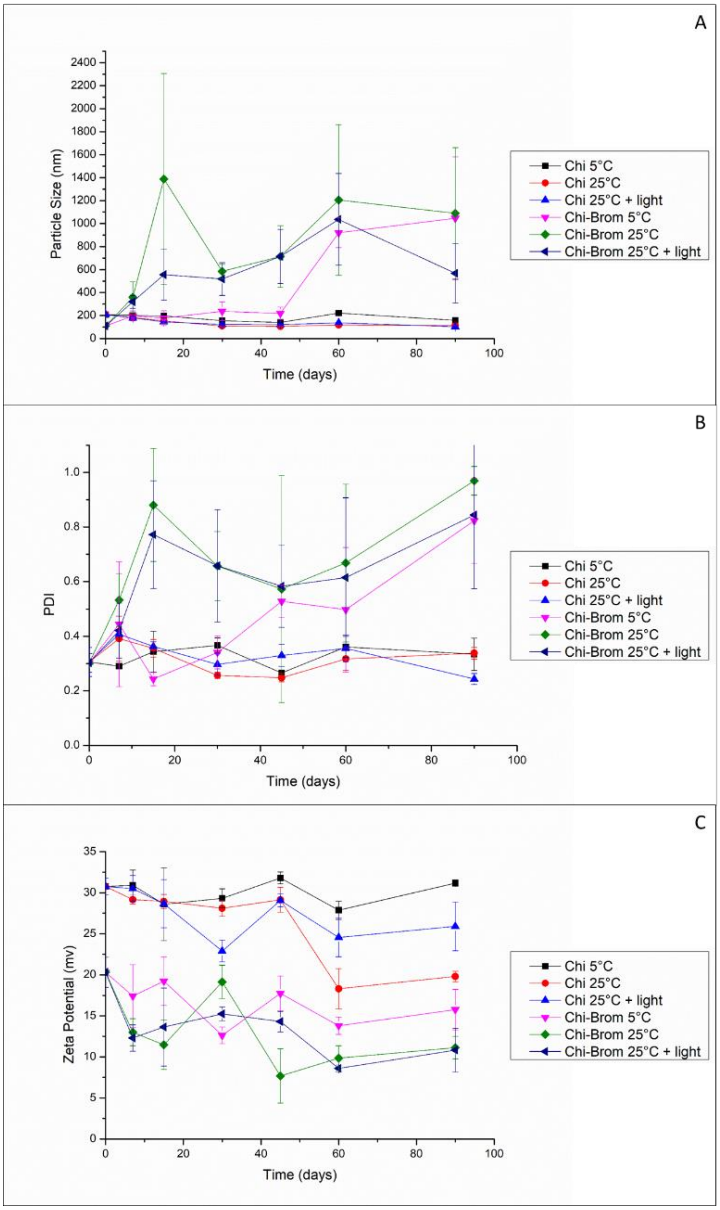

Figure V.4. Nanoparticles mean size (A), PDI (B) and zeta potential (C) during accelerated stability study. Chi = chitosan nanoparticles, Chi-brom = chitosan-bromelain nanoparticles. Error bars represent SD of three measurements.

Protein concentration and enzymatic activity were also determined during accelerated stability study. Chitosan nanoparticles did not present significant protein concentration and neither enzymatic activity in any studied time or condition. Chitosan-bromelain nanoparticles showed a decrease in protein concentration and enzymatic activity (Figure V.5) over the time, where enzymatic activity significantly decrease ($p < 0.05$, Student T-test) after 7 days. This decrease was slightly less pronounced when samples were stored in low temperatures in accordance with other studies using bromelain (Pereira et al., 2014; Spir et al., 2015; Lourenço et al., 2016). Given those results, chitosan-bromelain nanoparticles were unstable when stored

in liquid form, and thus freeze-drying is an important process for enhancing preservation of particle size for long-term storage (Fonte et al., 2016; Almalik et al., 2017) and bromelain enzymatic activity (Arakawa et al., 2001).

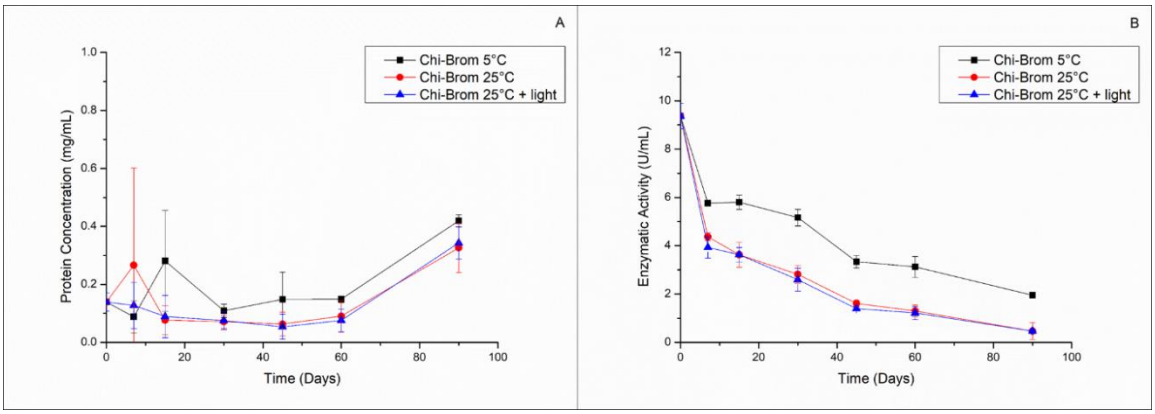

Figure V.5. Protein concentration (A) and enzymatic activity (B) in chitosan-bromelain nanoparticles solution during accelerated stability study. Chi-brom = chitosan-bromelain nanoparticles. Error bars represent SD of three measurements.

3.6. Nanoparticles freeze-drying

3.6.1. Lyoprotectants evaluation and collapse temperatures

Trehalose, maltose and glycine were chosen as possible lyoprotectants, and thus they were dissolved in chitosan-bromelain nanoparticles at 3% (w/v) concentration, to evaluate their effect on collapse temperature. The collapse temperature is the maximum allowable product temperature during primary drying, and its accurate determination is critical for freeze-drying process optimization (Pikal and Shah, 1990; Abdelwahed et al., 2006). Chitosan-bromelain nanoparticles showed a collapse temperature of -56 °C, which decreased to -49.3 °C, -34.8 °C and -28.0 °C with trehalose, maltose and glycine addition, respectively, determined by direct microscopic observation of collapse during freeze drying.

During primary drying (ice sublimation), the product should be below the collapse temperature in order to avoid product collapse and to prevent loss of macroscopic structure (Pikal and Shah, 1990; Abdelwahed et al., 2006; Fonte et al., 2016). However, drying a product below de collapse temperature carries a price, since the drying cycle is slower and more expensive at lower samples temperatures. In general, freeze-drying process below -40 °C is not practical (Carpenter et al., 1997; Tattini Jr et al., 2006), and thus, maltose and glycine were evaluated in factorial design as lyoprotectors, once they enable freeze-drying to occur at -40 °C.

3.6.2. Freeze-drying process

To validate a freeze-drying process, it is important to characterize the lyophilizate, the containing nanoparticles and the active content upon lyophilization and reconstitution (Fonte et al., 2016). A good freeze-drying process should result in a cake with good aspect and short reconstitution time (Williams and Polli, 1984; Fonte et al., 2016). Thus, the lyophilizate characterization should evaluate its aspect and reconstitution time. All lyophilizates had good aspects without shrinkage. Nanoparticles with glycine and maltose were immediately reconstituted after water addition, and resulting solutions were clear. Without lyoprotector, however, resuspension took more than 20 seconds and solutions a slightly turbidity, even after vortexing.

Lyophilizate powders were analysed by thermogravimetric analysis to determine residual moisture. Formulations with glycine at 3% and 5% concentrations presented 4.1% and 3.2% weight-loss respectively, while formulations with maltose presented 5.6% and 3.8%. Ideally, residual moisture should be lower than 2% (Abdelwahed et al., 2006; Sylvester et al., 2018), which was not observed for any dried-formulation.

A factorial design 2^3 was conducted in order to evaluate effects of lyophilization in nanoparticles suspension. Lyophilizates were reconstituted in distilled water to analyse particles physical characteristics by dynamic light scattering (

Table V.3) and encapsulation efficiency (Figure V.6), which were considered as outputs in the factorial design. After freeze-drying glycine at 3% (w/v) concentration was able to maintain a desirable polydispersity index, however zeta potential decrease. On the other hand, maltose presented an opposite behavior, increasing polydispersity and showing a less pronounced effect on zeta potential.

Table V.3. Nanoparticles size, PDI and zeta potential before and after freeze-drying process.

Nanoparticles		Z-ave (nm)	PDI	Zeta (mV)
None	**Before**	84.5 ± 6.1	0.231 ± 0.030	27.1 ± 5.1
	After	2386 ± 320.5	0.485 ± 0.043	20.2 ± 0.3
Glycine 3%	**Before**	93.7 ± 12.5	0.257 ± 0.020	28.6 ± 3.5
	After	161.7 ± 24.7	0.214 ± 0.025	15.7 ± 3.8
Glycine 5%	**Before**	94.2 ± 10.7	0.238 ± 0.006	30.0 ± 2.1
	After	223.6 ± 29.1	0.466 ± 0.004	18.6 ± 0.1
Maltose 3%	**Before**	74.5 ± 3.7	0.385 ± 0.039	19.7 ± 4.5
	After	90.2 ± 2.4	0.324 ± 0.019	23.0 ± 0.3
Maltose 5%	**Before**	70.6 ± 0.3	0.503 ± 0.006	20.5 ± 0.9
	After	76.8 ± 1.1	0.483 ± 0.019	21.6 ± 0.2

**Results presented as mean ± SD of two formulations, measured three times each. Z-ave = mean particle size, PDI = polydispersity index.*

An adequate freeze-drying process, that do no damages the nanoparticles integrity, allows the retention of the drug entrapped into nanoparticles, so it is important to quantify the active content upon freeze-dry (Fonte et al., 2016). Encapsulation efficiency increased in terms of protein concentration upon glycine and maltose addition, while enzymatic activity encapsulation decreased after glycine addition and increased after maltose addition (Figure V.6). After freeze-drying, it is possible to observe a slight increase in protein encapsulation efficiency for all samples. This behaviour may be due to under-quantification of free bromelain, which may be aggregated in the final product and unable to pass the membrane of ultrafiltration device. Enzymatic activity encapsulation also increased in all formulations.

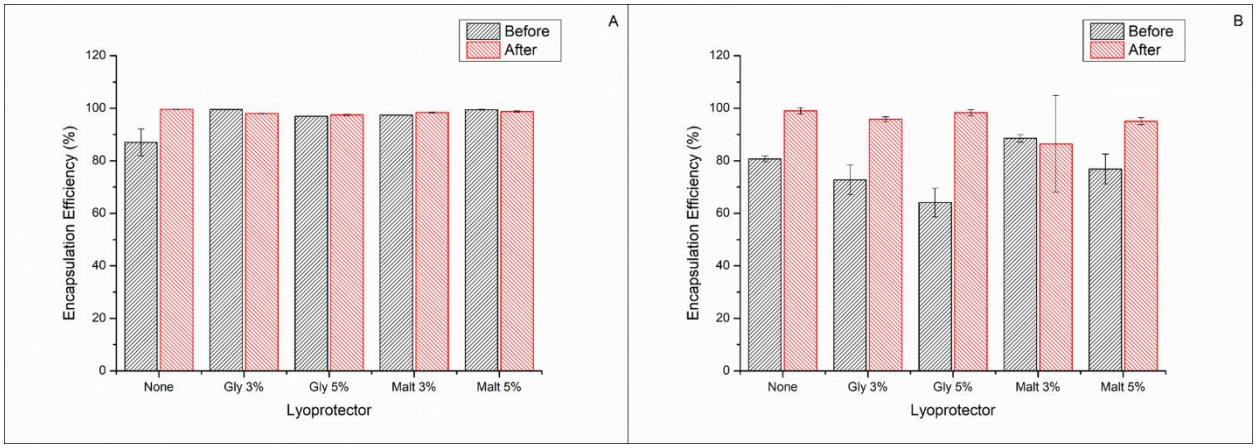

Figure V.6. Bromelain encapsulation efficiency into nanoparticles according with protein concentration (A) and enzymatic activity (B). Gly = glycine, Malt = maltose. Error bars represent SD of three measurements.

Outputs (mean particle size, PDI, D_{10}, D_{50}, D_{90}, zeta potential and encapsulation efficiency) were statistically analyzed using pareto diagrams (Figure V.7) and main effects of inputs in the outputs were also analyzed (Figure V.8). Lyoprotector significantly interfere in almost all outputs, excluding zeta potential and activity encapsulation. Glycine increases mean particle size by increase size percentiles 10, 50 and 90, however it also decrease polydispersity, indicating narrower size distribution. On the other hand, maltose decrease mean particle size and increase polydispersity, which is not an advantage. Lyoprotectors concentration was a significant input for polydispersity index, D_{10} and protein encapsulation, and lower concentrations (3%, w/v) decrease polydispersity, increasing D_{10} and protein encapsulation.

Excluding protein encapsulation, lyophilization significantly affects all outputs. The process increased mean particle size by increasing size percentile distribution, also increasing polydispersity and activity encapsulation. Zeta potential was decreased by freeze-drying process, which may cause some issue on particles stability that should be further evaluated. Considering all effects, formulations with glycine or maltose at 3% (w/v) concentration seems potential to better stabilize chitosan-bromelain nanoparticles, and further stability studies are necessary.

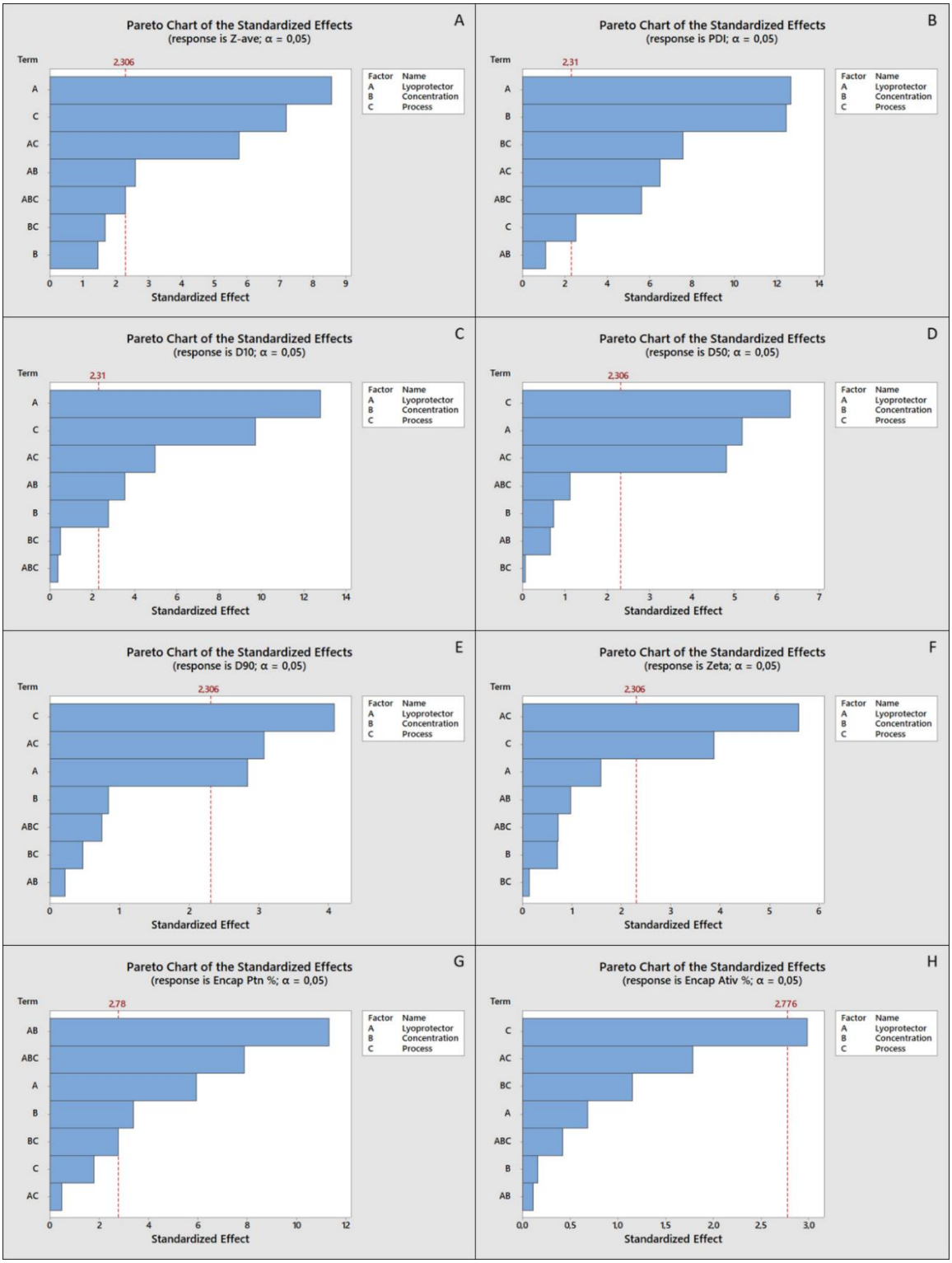

Figure V.7. Pareto chart of factorial design relating the studied inputs to mean particle size (A), polidispersity index (B), D_{10} (C), D_{50} (D), D_{90} (E), zeta potential (F) and encapsulation efficiency in terms of proteins (G) and enzymatic activity (H).

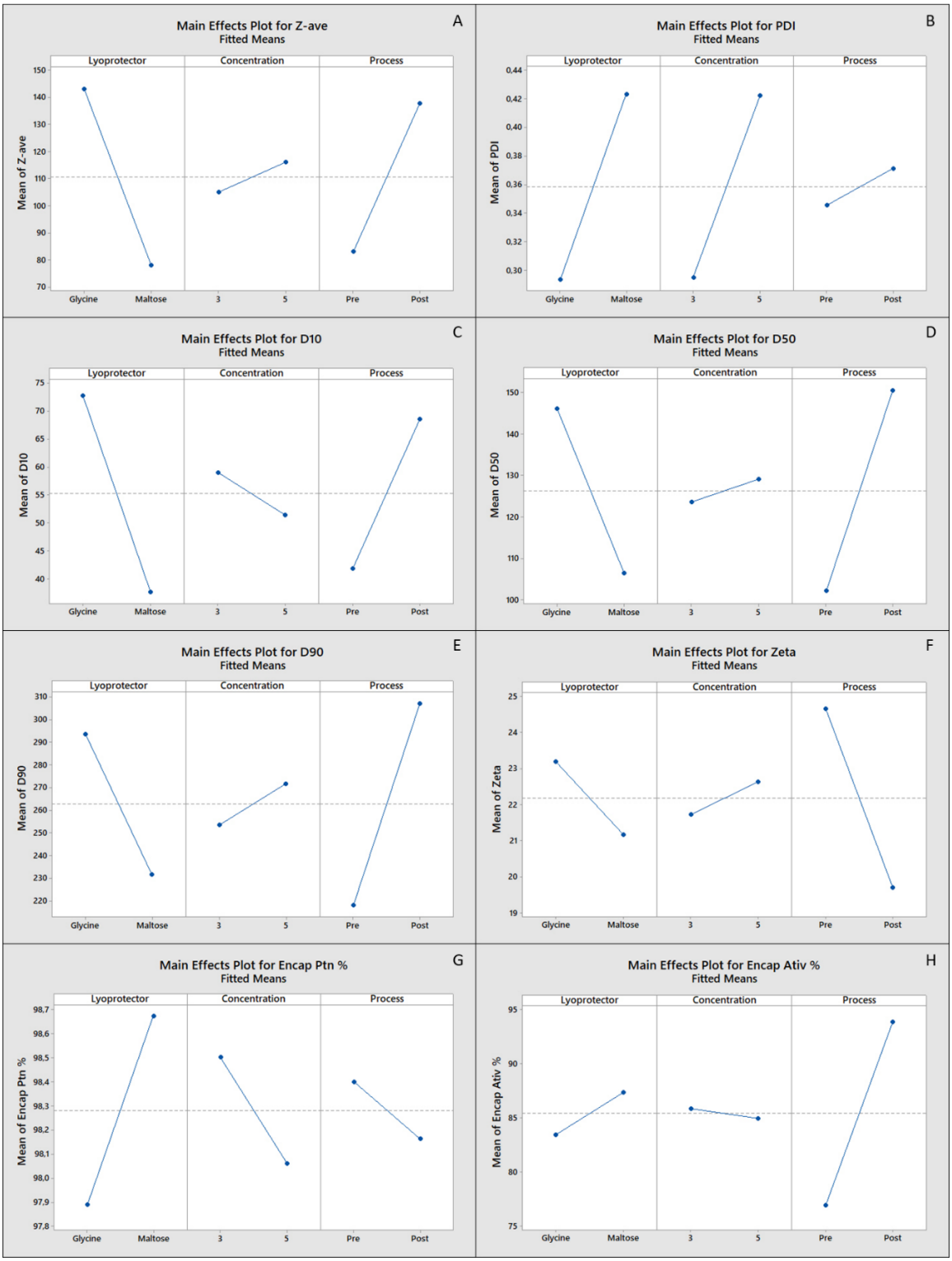

Figure V.8. Main effect plots of factorial design for mean particle size (A), polidispersity index (B), D_{10} (C), D_{50} (D), D_{90} (E), zeta potential (F) and encapsulation efficiency in terms of proteins (G) and enzymatic activity (H).

4. Conclusions

Chitosan-bromelain nanoparticles were produced by ionic gelation method, with mean particle size of 100.9 ± 0.5 nm. These nanoparticles have an adequate polydispersity, of 0.222 ± 0.012, in addition to satisfactory charge stability, with a zeta potential of +21.9 ± 0.5 mV. There is a good bromelain encapsulation efficiency, of 87,0% according to total proteins concentration, and 80,7% according to the enzymatic activity. However, nanoparticle did not present desired stability in aqueous suspension, and nanoparticles were freeze-dried. After freeze-drying process, glycine and maltose at 3% concentration showed promising results for nanoparticles formulation, with desirable nanoparticles properties and encapsulation efficiency. Further stability studies with both formulations are necessary.

5. Acknowledgements

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CAPÍTULO VI. "ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF FREE AND CHITOSAN-ENCAPSULATED BROMELAIN"

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Abstract

Recently, a wide range of therapeutic benefits have been attributed to bromelain such as enzymatic debridement of necrotic tissues from ulcers and burn wounds, anti-inflammatory activities, and also antitumor and antioxidant properties. However, its mode of action is not yet fully understood, and there remains a need for studies investigating the mechanism of action and functional properties of bromelain. To overcome bromelain stability issues, chitosan-bromelain nanoparticles were formulated. Free bromelain, chitosan-bromelain nanoparticles and chitosan nanoparticles were studied for *in vitro* antioxidant activity, antiproliferative activity and also cell migration and proliferation in scratch assay. Antioxidant activity was investigated against DPPH and ABTS radicals. Free bromelain antioxidant activity was found to be concentration and time-dependent, and after encapsulation, its activity remained, being delayed and dependent of its release. Antiproliferative activity were investigated on eight human tumor cell lines and one human non tumor cell line (HaCat), using doxorubicin as a positive control. Free bromelain inhibited growth of six tumor cell lines (U251, MCF-7, PC-3, OVCAR-03, HT-29, K562) while chitosan-bromelain inhibit only one cell line growth (K562), after 48 h treatment. After 144 h treatment, free and encapsulated bromelain totally inhibited glioma cell growth in the same concentration, showing that bromelain activity remained after encapsulation process and was dependent on its release. Although some antiproliferative activity was observed, free and encapsulated bromelain did not show toxicity on human keratinocytes, enabling its use as a topical active. Scratch assay was conducted using HaCat cells, and bromelain showed lower wound retraction when compared with all other samples. Chitosan and chitosan-bromelain nanoparticles achieved more than 90% wound retraction after 24 hours. These results indicate that bromelain was efficiently encapsulated in chitosan nanoparticles, and encapsulation process did not interfere in bromelain activities, and enhanced its wound retraction action. In addition, nanoencapsulation seems to provide some protection for bromelain, once antioxidant and antiproliferative activities were delayed or dependent of a higher concentration for nanoparticles suspensions.

Keywords: bromelain; chitosan-encapsulation; antiproliferative assay; antioxidant assay; scratch assay

1. Introduction

Ananas comosus L., popular known as pineapple, has been used for centuries as a folk medicine by the indigenous inhabitants of Central and South America, to treat a range of ailments, mainly as a digestive aid and a wound healing agent (Amini et al., 2013; Gani et al., 2015). Its medicinal properties are attributed to bromelain, a mixture of proteolytic enzymes (or proteases) and non-protease components, including phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates (Chobotova et al., 2010).

Different studies claim a wide range of medical applications for bromelain, such as inhibitory properties for platelet aggregation, antioxidant and anti-inflammatory action, antibacterial and antifungal activities, fibrinolytic activity, modulation of cytokines and immunity, enhanced absorption of other drugs, skin debridement, digestive assistant, enhanced wound healing and anti-carcinogenic action (Maurer, 2001; Bala et al., 2012; Dutta and Bhattacharyya, 2013; De Lencastre Novaes et al., 2016; Muhammad and Ahmad, 2017; Ramli et al., 2017). Bromelain is already sold in health stores in the United States and Europe, as a nutritional supplement to promote digestive health, wounds healing, and as an anti-inflammatory agent (Taussig and Batkin, 1988; Romano et al., 2014). Particular attention has been given to bromelain antioxidant (Manosroi et al., 2014) and antiproliferative activities (Chobotova et al., 2010; Amini et al., 2013; Bhatnagar et al., 2014; Romano et al., 2014; Gani et al., 2015; Bhatnagar et al., 2016; Oliveira et al., 2017), and to its wound healing improvement (Bromelain. Monograph, 2010; De Lencastre Novaes et al., 2016).

Nanotechnology is an emerging field that is potentially changing the way of treating diseases through drug delivery systems, which primarily aims to improve the bioavailability of chemotherapeutic agents and to reduce their adverse side effects, ultimately leading to improved drug efficacy (Bhatnagar et al., 2016). The nanoparticulate systems offer many other advantages, such as improving bioavailability, extending the therapeutic effect of the drug at the target site, and improving the stability of the drug against chemical and enzymatic degradation (Hamidi et al., 2008).

Particularly, therapeutic proteins present a challenge for drug therapy, especially due to its immunogenicity and inflammatory potential, and physical and chemical degradation (Mao et al., 2010; Pachioni-Vasconcelos et al., 2016). Therefore, the use of nanotechnology to delivery protein drugs seems a possible strategy to obtain safe and effective therapeutic protein preparations, and a to stabilise protein drugs against denaturation by enzymatic digestion, increasing their biopharmaceutical applications (Prego et al., 2006; Antosova et al.; Balcão et al., 2013).

Chitosan is a naturally occurring and abundantly available polysaccharide, which presents excellent biocompatibility and biodegradability. Because of its favorable characteristics, chitosan nanoparticles have been extensively studied for drug delivery systems (Sinha et al., 2004). They have been prepared through various methods, but ionic gelation is often favoured as it is a simple, mild, and controllable process (O'callaghan and Kerry, 2016).

In the present study, a series of *in vitro* assays were performed to investigate bromelain efficacy before and after nanoencapsulation process, in an attempt to understand the modifications that this process can lead in a bioactive product. For this, we investigated *in vitro* antioxidant activity, antiproliferative effect on tumor and non-tumor cell lines, and keratinocytes migration of bromelain and chitosan-bromelain nanoparticles.

2. Material and Methods

2.1. Materials

Bromelain from pineapple stem, low molecular weight chitosan, azocasein and Bradford reagent were purchased from Sigma-Aldrich (Sao Paulo, Brazil). All other reagents were purchased at analytical grade.

2.2. Bromelain solution

Bromelain solution (10 mg/mL) was prepared by diluting bromelain from pineapple steam in distilled water. Prior to use, the solution was filtered through a 0.22μm filter. Protein concentration and enzymatic activity for bromelain were determined according with Bradford and Azocasein methods respectively (Bradford, 1976; Coelho et al., 2016).

2.3. Chitosan and chitosan-bromelain nanoparticles formulation

Nanoparticles were produced by the ionic gelation method (Shu and Zhu, 2000; Servat-Medina et al., 2015; Goycoolea et al., 2016) using sodium tripolyphosphate (TPP) as crosslinking agent. Briefly, TPP solution (0.5 mg/mL in distilled water and filtered at 0.22 μm, 3 mL) was dropwise on the chitosan solution (2.5 mg/mL in 1% (v/v) acetic acid at pH 5.0 and filtered at 0.45 μm, 2 mL). Immediately after that, the bromelain solution (1 mL) or 0.22 μm filtered-water (1 mL) was added and mixed under magnetic stirring (Fisatom, Mod 753E, Sao Paulo, Brazil) at 350 rpm for 40 minutes to afford chitosan-bromelin or empty chitosan nanoparticles, respectively. Nanoparticles physicochemical parameters of average particle size, polydispersity index and zeta potential were determined using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipment.

2.4. In vitro antioxidant activity

The tests to determine the antioxidant capacity have peculiarities as their way of assessing such activity, and two widely used methods to assess this potential were chosen: scavenging capacity of radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6 sulfonic acid (ABTS) (Brand-Williams et al., 1995; Re et al., 1999; Nile et al., 2012). Five different concentrations were prepared successively diluting tested samples (bromelain solution, chitosan-bromelain and chitosan nanoparticles) in distilled water affording final concentrations of 6.25%, 12.5%, 25%, 50% and 100% (v/v). In general, 2.5 mL of sample was mixed with 2.5 mL of DPPH radical solution, mixed, incubated for 30 min, and read at 531 nm. For ABTS assay, 30 µL of sample was mixed with 3 mL of radical, vortexed for 6 minutes and read at 734 nm. After first measurement, samples were kept closed and protected from light and absorbance was once again read after 24h. Water was used for negative control and maximum absorbance. The free radical sequestering capacity was calculated in relation to the absorbance of radicals' solution with water, as follows:

$$\%Inhibition = \frac{Abs_{water} - Abs_{sample}}{Abs_{water}} x100 \qquad \text{Equation I}$$

2.5. In vitro assays

2.5.1. Cell lines

A panel of eight human tumor cell lines [U251 (glioma), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype of multiple drugs resistance), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), HT-29 (colon adenocarcinoma) and K-562 (chronic myeloid leukemia)], kindly provided by Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA, was used in the antiproliferative assay. The human non-tumor cell line HaCat (keratinocyte), provided by Dr. Ricardo Della Coletta (University of Campinas-UNICAMP, Brazil), was used in antiproliferative and scratch assays. For the experiments, all cell lines were used between passages 5 to 12.

Stock cultures were grown in 5 mL of RPMI-1640 supplemented with 5% fetal bovine serum (RPMI/FBS 5%) and 1% penicillin:streptomycin mixture (1000 U/mL:1000 µg/mL) (complete medium) at 37 °C and 5% of CO_2 . For the scratch assay, samples were diluted in RPMI-1640 supplemented with 0.2% fetal bovine serum and 1% penicillin:streptomycin mixture (1000 U/mL:1000 µg/mL) (scratch medium).

2.5.2. Sample preparation

Bromelain, chitosan-bromelain and chitosan nanoparticles stock solutions (5 mg/mL) were prepared with water, and then successively diluted with complete (antiproliferative assay) or scratch (scratch assay) media, achieving final concentration of 0.25, 2.5, 25 and 250 μg/mL (antiproliferative assay) and 250 μg/mL (scratch assay). Doxorubicin (0.025, 0.25, 2.5 and 25 μg/mL) and complete medium were used as positive controls in antiproliferative and scratch assays, respectively.

2.5.3. Antiproliferative assay

Cells in 96-well plates (100 μL cells/well) were exposed to different concentrations of samples (0.25, 2.5, 25 and 250 μg/mL) in triplicate, for 48 h at 37 °C and 5% of CO2. Before (T0 plate) and after (T1 plates) sample addition, cells were fixed with 50% trichloroacetic acid (50 μL/well) and cell proliferation was determined by protein quantitation with sulforhodamine B at 540 nm (Monks et al., 1991; Bachiega et al., 2016; Nunes et al., 2017). The GI50 values (concentration that inhibits 50% cell growth or cytostatic effect) were determined through sigmoidal regression using Origin 8.0® software (OriginLab Corporation).

2.5.4. Scratch assay

For the scratch assay, HaCat cells were plated with complete medium in 12-well plate and incubated, for at least 24 h, at 37 °C and 5% of CO2. After reaching confluence, wound was performed as one straight line on each well made with the sterile p200 pipet tip followed by medium removal. After washing each well with scratch medium (1 mL/well) to remove debris, cells were treated with complete medium (5.0% of fetal bovine serum, positive control) or samples diluted in scratch medium. The follow-up of the wound closure was observed in an inverted phase microscope at 0, 9, 18 and 24 hours (Todaro et al., 1965; Liang et al., 2007). Samples were tested in duplicate and three images were taken of each well. The images acquired for each sample, at different times, were quantitatively analyzed using ImageJ software.

3. Results and Discussion

3.1. Chitosan and chitosan-bromelain nanoparticles

Chitosan and chitosan-bromelain nanoparticles were successfully produced by ionic gelation method and characterized (Table VI.1). Bromelain incorporation promoted a decrease in average particle size and zeta potential in comparison to empty chitosan nanoparticles. These results can be attributed to the negative charge of bromelain surface at pH 5.0 (Hebbar et al.,

2012) which improve the electrostatic interaction between chitosan and bromelain. More, chitosan-bromelain nanoparticles showed a slight increase in the polydispersity index (PDI). Considering PDI criteria, both nanoparticles can be considered as moderately polydisperse, as experimental PDI values were between 0.1 and 0.4 (Bhattacharjee, 2016).

Table VI.1. Chitosan and chitosan-bromelain nanoparticles characterization by dynamic light scattering and zeta potential.

Nanoparticle	Average Size (nm)	PDI	Zeta Potential (mV)
Chitosan	114.6 ± 1.9	0.217 ± 0.031	+23.5 ± 2.0
Chitosan-Bromelain	93.4 ± 0.1	0.226 ± 0.008	+23.4 ± 2.7

**Results presented as mean ± SD of three measurements.*

Further, protein concentration (Bradford, 1976) and enzymatic activity (Coelho et al., 2016) were determined for free bromelain, chitosan and chitosan-bromelain nanoparticles solutions (Table VI.2). As expected, chitosan nanoparticles showed absorbance values similar for blank sample in both evaluations (data not shown) demonstrating that whatever was observed to chitosan-bromelain nanoparticles can be attributed to bromelain. Thus, the low levels of protein concentration and enzymatic activity observed for nanoencapsulated bromelain confirming that bromelain was encapsulated with chitosan been less available to interact with the colorimetric assays.

Table VI.2. Protein concentration and enzymatic activity of free and encapsulated bromelain.

Bromelain	Protein Concentration (mg/mL)	Enzymatic Activity (U/mL)
Free	1.70 ± 0.30	18.44 ± 0.04
Nanoencapsulated	0.53 ± 0.47	8.54 ± 0.90

**Results presented as mean ± SD of three measurements.*

3.2. In vitro antioxidant activity

Bromelain antioxidant activity as free radical scavenging against DPPH have been previously reported (Manosroi et al., 2014; Ataide et al., 2017). In this study, the antioxidant activity of free bromelain, chitosan and chitosan-bromelain nanoparticles solutions were evaluated using DPPH (Figure VI.1A) and ABTS (Figure VI.1B) radicals. As previously reported, free bromelain presented antioxidant activity against DPPH, which was dependent on

the tested concentration and time, as can be noted that after 24 h, antioxidant activity increased. When using ABTS radical, free bromelain did not show a significant antioxidant activity right after normal reaction time. After 24 h, however, samples were spectrophotometrically read once more, and antioxidant activity was concentration-dependant, reaching around 88% for bromelain highest concentration.

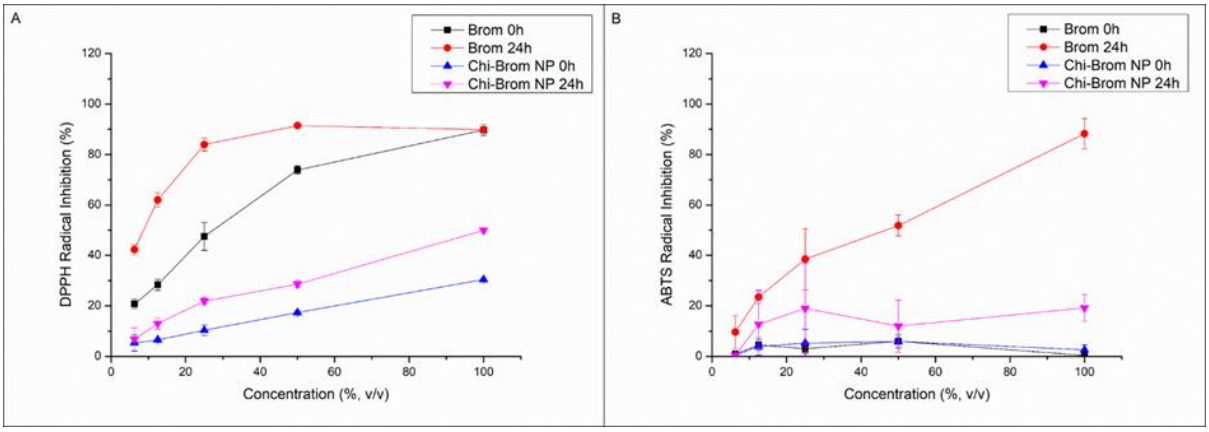

Figure VI.1. Antioxidant activity using DPPH (A) and ABTS (B) radicals. Brom = bromelain solution; Chi-Brom NP = chitosan-bromelain nanoparticles; Chi-NP = chitosan nanoparticles.

Chitosan nanoparticles present a small inhibition of DPPH and ABTS, thus its absorbance was considered and deducted from chitosan-bromelain nanoparticles absorbance. Chitosan-bromelain nanoparticles presented around 14% and 40% DPPH inhibition in the lowest and highest concentration, respectively. This inhibition was lower than that observed for free bromelain (around 21% and 89% for the lowest and highest concentration, respectively). After 24 h, DPPH inhibition increased to 57% in the highest tested concentration, which may be attributed to bromelain release from nanoparticles. The same behavior was observed against ABTS radical. In the first measurement no significant inhibition was reported, however after 24 h, 5% of inhibition was observed in the lowest concentration, and an inhibition between 31% and 40% was observed in other concentrations. Both results serve as an indicative of bromelain incorporation in the nanoparticles structure. They also collaborate to affirm that after encapsulated, bromelain maintained its activity.

3.3. In vitro antiproliferative assay

The antiproliferative activity of free bromelain, chitosan and chitosan-bromelain nanoparticles was assessed through GI_{50} (concentration that inhibits 50% cell growth or cytostatic effect) parameter against eight human tumour cell lines of different histological and

genetic origins, and one human non-tumor cell line (Table VI.3, Figure VI.2). The chitosan and chitosan-bromelain nanoparticles solutions were prepared considering an equivalence to the final bromelain concentration. Thus, bromelain showed a weak cytostatic effect against glioma cells (U251) that was supressed by chitosan encapsulation (Table VI.3). Moreover, either bromelain solution neither nanoparticles formulations showed antiproliferative effect against HaCat (human keratinocytes) suggesting that there was a safe limit to its topical use.

As it is possible to note, bromelain solution was able to inhibit growth of six cell lines (glioma, breast, prostate, ovarian, colon adenocarcinoma and chronic myeloid leukemia) while chitosan-bromelain nanoparticles were able only to inhibit leukemia cells growth. For leukemia cells, GI_{50} was 60.7 µg/mL and 204.4 µg/mL for free and encapsulated bromelain, respectively. Free bromelain displayed a weak growth inhibition of glioma cells (GI_{50} = 44.9 µg/mL), however after nanoencapsulation this activity was not observed even in the highest tested dose. Based on those results, it is possible to conclude that the nanoencapsulation process immobilized bromelain, decreasing its antiproliferative effect, which was dependent on higher doses.

Table VI.3. GI_{50} values in µg/mL of the in vitro antiproliferative activity of doxorubicin, bromelain solution, chitosan-bromelain nanoparticles and chitosan nanoparticles.

	2*	**m***	**a***	**4***	**p***	**o***	**h***	**k***	**q****
Doxorubicin	<0.025	<0.025	0.24	<0.025	0.23	0.057	0.13	0.031	<0.025
Bromelain	44.9	160.0	>250	>250	139.5	95.2	220.4	60.7	>250
Chi-Brom NP	>250	>250	>250	>250	>250	>250	>250	204.4	>250
Chi NP	250	>250	>250	>250	>250	>250	>250	>250	>250

**Human tumor cell lines: 2 = U251 (glioma); m = MCF-7 (breast); a = NCI-ADR/RES ((ovarian expressing phenotype of multiple drugs resistance); 4 = NCI-H460 (lung, non-small cells); p = PC-3 (prostate); o = OVCAR-03 (ovarian); h = HT-29 (colon adenocarcinoma); k = K562 (chronic myeloid leukemia). **Human non-tumor cell line: q = HaCaT (keratinocyte). Chi-Brom NP = chitosan-bromelain nanoparticles; Chi-NP = chitosan nanoparticles.*

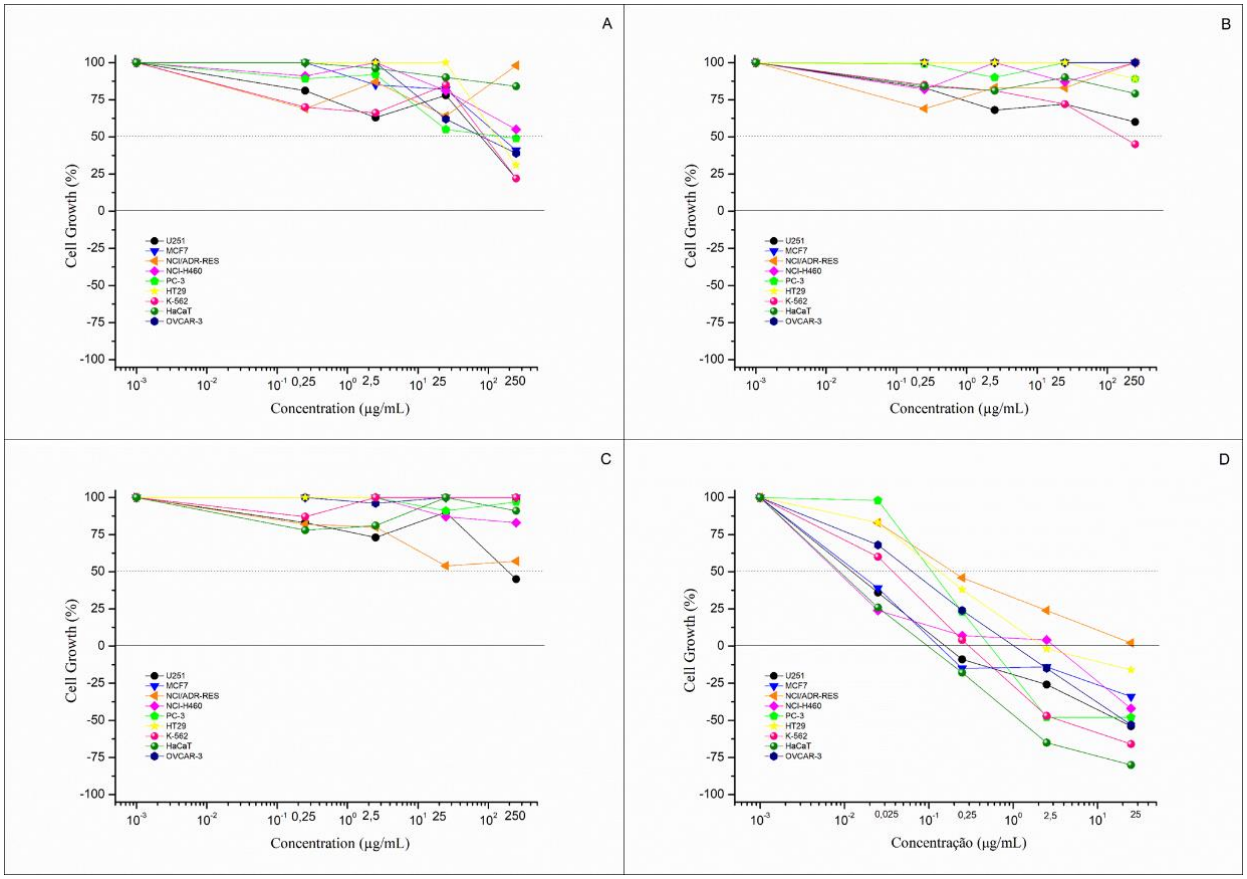

Figure VI.2. Antiproliferative activity of free bromelain solution (A), chitosan-bromelain nanoparticles (B), chitosan nanoparticles (C), and doxorubicin (D) after 48 h exposition.

According to literature, the wall material is a key factor in drug availability from a nanoparticle. Thus, Eudragit-coated bromelain PLGA nanoparticles and Bromelain-functionalized multiple-wall lipid-core nanocapsules were more active in in vitro evaluations with different cell lines than free bromelain (Bhatnagar et al., 2014; Bhatnagar et al., 2016; Oliveira et al., 2017). More, these authors also reported that time incubation was directly correlated with cytotoxic effect (higher time, higher effect) (Bhatnagar et al., 2014; Bhatnagar et al., 2016).

Our results (Table VI.3) pointed out that instead chitosan delayed bromelain release in culture medium. Bearing this context in mind, we evaluated the cytostatic effect of bromelain, chitosan-bromelain and chitosan nanoparticles solution against HaCat (keratinocytes) and U251 (glioma) cell lines after 144h incubation (Table VI.4, Figure VI.3). As after 48h, empty chitosan nanoparticles were not able to cause any antiproliferative effect even after 144 h, proven that nanoparticles formulation does not have any antiproliferative effect itself. More, after long exposition, free and encapsulated bromelain were able to total inhibit U251 growth, and as so the total growth inhibition (TGI) dose was calculated (Table VI.4). Thus, increasing time

exposition resulted in higher antiproliferative effect as described in literature for others bromelain-nanoparticle systems. Considering the effect on non-tumor HaCat cells, the increasing time exposition afforded a weak antiproliferative activity of free bromelain (TGI = 119.33 μg/mL). However, after nanoencapsulation process, this effect was not observed (TGI > 250 μg/mL, Table VI.4).

Table VI.4. TGI values in μg/mL of the in vitro antiproliferative activity of doxorubicin, bromelain solution, chitosan-bromelain nanoparticles and chitosan nanoparticles.

	2*	q**
Doxorubicin	0.25	0.25
Bromelain	0.25	119.33
Chi-Brom NP	0.25	>250
Chi NP	>250	>250

**Human tumor cell line: 2 = U251 (glioma). **Human non-tumor cell line: q = HaCaT (keratinocyte). Chi-Brom NP = chitosan-bromelain nanoparticles; Chi-NP = chitosan nanoparticles.*

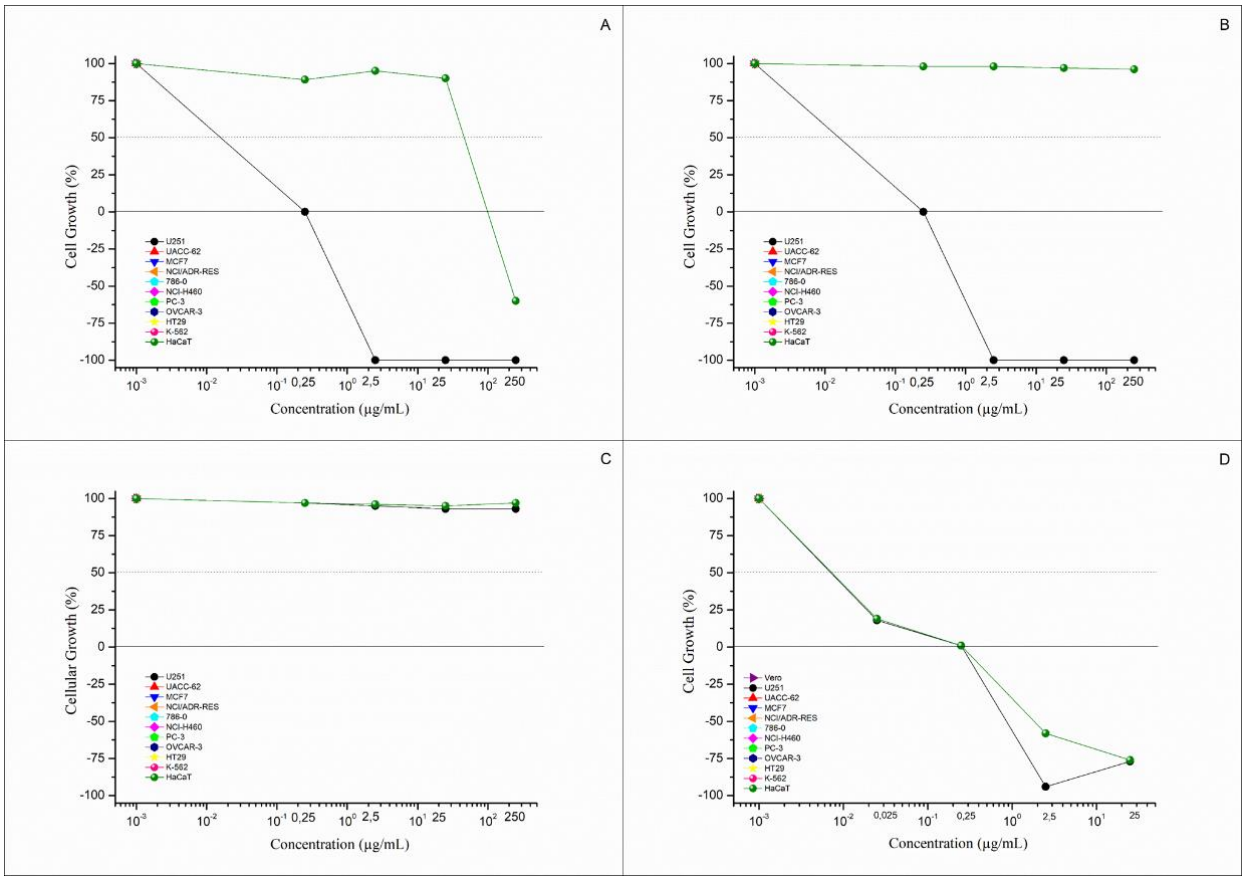

Figure VI.3. Antiproliferative activity of free bromelain solution (A), chitosan-bromelain nanoparticles (B), chitosan nanoparticles (C), and doxorubicin (D), after 144 h exposition.

3.4. In vitro scratch assay

In vitro scratch wound assay was carried out to observe the effects of free and encapsulated bromelain on the healing process. Scratch medium and scratch medium supplemented with 5% FBS were used as controls, and total wound closure was achieved in 24 and 18h, respectively (Figure 4). Previous studies have already report chitosan activity as wound healing promotor (Dai et al., 2011; Felice et al., 2015). Howling et al. (2001) had also reported that high chitosan with high deacetylation degrees strongly stimulates fibroblasts proliferation when compared with low deacetylation degree. In this study, chitosan nanoparticles promoted 46% of wound retraction after 9h, reaching 94% after 24 hours. The same behavior was observed for chitosan-bromelain nanoparticles, promoting 46% and 97% retraction after 9 and 24 hours, respectively.

In its turn, free bromelain did not present promising results, showing around 27% of scratch retraction after 9 hours, which was lower than all other samples. After 18 hours, bromelain closed around 34% of wound area and started causing HaCat cell death, evidenced by the presence of granules in micrographs and confluency loss. Aichele et al. (2013) showed that bromelain moderately attenuated endothelial cell and fibroblast proliferation. Authors concluded that this attenuation was caused by driving cells into the resting state of the cells, and not by apoptosis. In this same study, bromelain was not able to influence endothelial cells migration, and slowed down fibroblasts migration under hypoxia. This results may indicate that bromelain action in wound healing cannot be attributed to keratinocytes proliferation or migration, it rather may be due to its debriding action, which in vivo remove cell debris and necrotic tissues, in accordance with other studies (Singer et al., 2010; Wu et al., 2012).

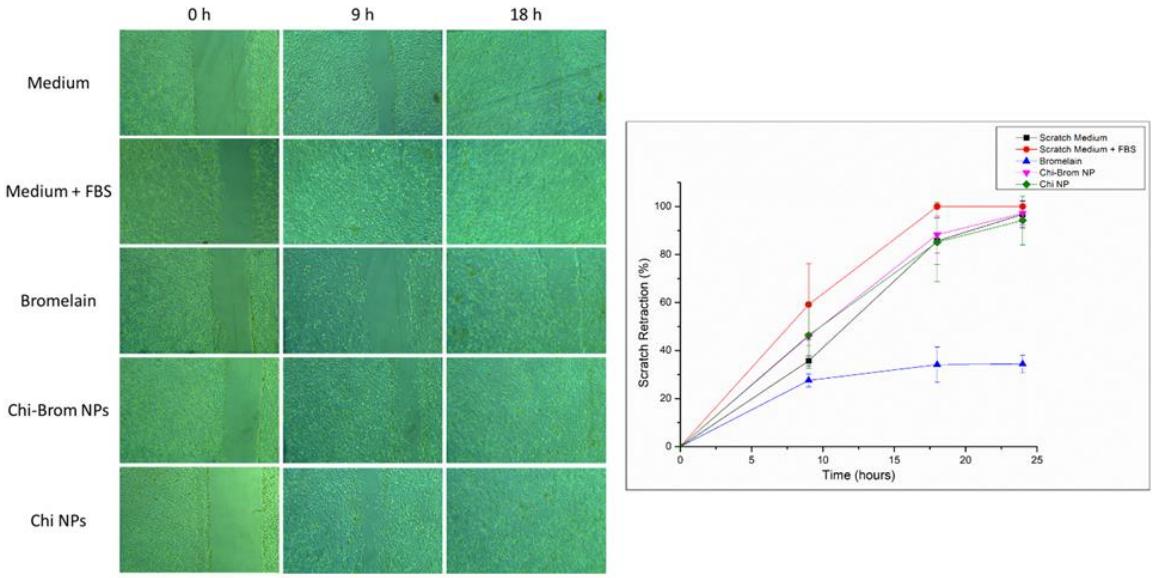

Figure VI.4. Representative micrographs of HaCat cells treated with 250 µg/mL of controls and samples for 0, 9 and 18 hours. Graph represents quantification of the effects of controls and samples on scratch retraction during assay period. FBS = fetal bovine serum; Chi-Brom NPs = chitosan-bromelain nanoparticles; Chi NPs = chitosan nanoparticles.

4. Conclusions

Free bromelain presented time and dose-dependent antioxidant activity against DPPH and ABTS radicals, which was also observed in a lower mode after bromelain nanoencapsulation, showing that bromelain was incorporated in the nanoparticle structure. Free bromelain presented antiproliferative effect against six tumor cell lines, while encapsulated bromelain was able to kept the effect against only one tumor cell line, and this effect was dependent on a higher dose. After exposure to treatment for 144 h, free and encapsulated bromelain were able to totally inhibit glioma cell growth. In the highest tested concentration, free bromelain was able to inhibit keratinocyte growth, however this effect was not observed for encapsulated bromelain, which corroborates its potential application as topical and healing agent. In scratch assay, bromelain showed low activity, which was enhanced after bromelain encapsulation, achieving more than 90% of wound retraction after 24 hours. These results indicate that bromelain was efficiently encapsulated in chitosan nanoparticles, and encapsulation process did not interfere in antioxidant and antiproliferative bromelain activities, which were delayed or dependent on higher doses; and enhanced its wound retraction action. In addition, nanoencapsulation seems to provide some protection for bromelain, once antioxidant and antiproliferative activities were delayed or dependent of a higher concentration for nanoparticles suspensions.

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4. DISCUSSÃO GERAL

Feridas são definidas como uma “ruptura da estrutura e função anatômica normal da pele” de acordo com a *Wound Healing Society*. De acordo com a mesma sociedade, essa lesão tecidual resulta na "perda de continuidade do epitélio com ou sem perda de tecido conjuntivo subjacente” (Lazarus *et al.*, 1994). As feridas podem ser classificadas em duas categorias principais: agudas e crônicas. As feridas agudas são lesões que seguem um processo de cicatrização ordenado e o completam dentro do prazo esperado, sendo causadas tipicamente por cortes ou incisões cirúrgicas. Enquanto as feridas crônicas, são aquelas que cicatrizam lentamente devido a lesões repetidas ao tecido ou a outras fisiopatologias que interferem no tempo e/ou na sequência ordenada do processo de cicatrização (Lazarus *et al.*, 1994; Strodtbeck, 2001).

Por sua vez, a cicatrização é definida como "um processo dinâmico complexo que resulta na restauração da continuidade e função anatômica da pele" (Lazarus *et al.*, 1994), sendo considerada um processo biológico regular no corpo humano, uma vez que a pele tem a habilidade natural de promover sua regeneração após lesionada (Guo and Dipietro, 2010; Pereira and Bártolo, 2016). No entanto, esse processo regular depende de muitos fatores e pode ser comprometido sob certas condições, como por exemplo em pacientes com diabetes e em pacientes com queimaduras extensas e de grau elevado (Beldon, 2010; Groeber *et al.*, 2011).

Um processo de cicatrização inadequado pode levar a cronificação da ferida, aumentando o risco de infecções e afetando a saúde e a qualidade de vida do paciente, com potencial morbidade e mortalidade, além de um efeito estético final ruim (Abdelrahman and Newton, 2011; Young and Mcnaught, 2011). Para evitar possíveis complicações, as feridas devem ser corretamente manejadas, principalmente com o uso adequado da farmacoterapia tópica e de curativos (Fraser *et al.*, 2004; Atiyeh *et al.*, 2007; Gueldner *et al.*, 2017).

Uma revisão da literatura com as principais fases do processo de cicatrização e os principais ativos sintéticos foi conduzida neste trabalho (Capítulo I). De acordo com os estudos encontrados na literatura científica, o complexo e intricado processo de cicatrização pode ser acelerado e aprimorado através do uso de técnicas de curativo, produtos e ativos tópicos. Nesse cenário, o tratamento para feridas, principalmente as crônicas, torna-se um alvo importante para o desenvolvimento tecnológico médico-farmacêutico.

A natureza é uma fonte de tratamentos medicinais, com o uso de plantas como protótipos para o desenvolvimento de medicamentos e para a extração de compostos ativos. Os compostos de origem natural tem sido utilizados no cuidado da pele principalmente devido às

propriedades anti-inflamatória, antimicrobiana e estimuladora de proliferação e migração celular (Pereira and Bártolo, 2016). Com base na tendência do uso de compostos de origem natural para a cicatrização, uma revisão da literatura disponível sobre as características e mecanismos de ação de compostos naturais foi realizada, selecionando-se artigos com menos de 10 anos e escolhendo os ativos com base em seu uso popular (Capítulo II).

Muitos estudos selecionados apresentaram o uso bem sucedido de compostos naturais na indução de proliferação e migração de células *in vitro*, e boa re-epitelização usando modelos *in vivo*. Esses estudos demonstraram a aplicação potencial de extratos naturais e seus ativos isolados para o desenvolvimento de formulações visando a sua aplicação no manejo de feridas.

Dentre os ativos naturais com potencial aplicação na cicatrização, podemos destacar a bromelina, que é um complexo de proteases e outras substâncias não-enzimáticas encontradas principalmente no abacaxi (*Ananas comosus* L.) e em outras espécies da família Bromeliaceae (Maurer, 2001). Apesar de não ter seu mecanismo molecular de ação completamente identificado, a bromelina tem ganho grande aceitabilidade como agente fitoterápico, devido às suas reconhecidas propriedades anti-inflamatória, antitrombótica e fibrinolítica, atividade antitumoral e efeito imunomodulador (Taussig and Batkin, 1988; De Lencastre Novaes *et al.*, 2016; Rathnavelu *et al.*, 2016; Ataide *et al.*, 2017). É importante ressaltar, que a bromelina é vendida nos Estados Unidos e na Europa como suplemento nutricional para promoção da digestão e cicatrização de feridas, e como agente anti-inflamatório (Romano *et al.*, 2014; Muhammad and Ahmad, 2017).

Estudos anteriores apontam para a instabilidade da bromelina quando incorporada diretamente em formulações de uso tópico, como emulsões e géis, mesmo com seu armazenamento em refrigerador (Pereira *et al.*, 2014; Spir *et al.*, 2015; Lourenço *et al.*, 2016). Muitas enzimas e proteínas são frágeis e mesmo pequenas mudanças conformacionais podem reduzir sua atividade (Balcão *et al.*, 2013). Por isso, o uso de proteínas como ativos em formulações farmacêuticas é desafiador, e vários métodos tem sido utilizados a fim de aumentar sua estabilidade (Pachioni-Vasconcelos *et al.*, 2016). Grande atenção tem sido dada ao uso de nanopartículas, uma vez que além de aumentar a estabilidade do ativo, esses sistemas conseguem driblar outros desafios como baixa solubilidade, baixa permeabilidade e elevada toxicidade (Kammona and Kiparissides, 2012; Bernkop-Schnürch, 2013).

As bases de dados *Web of Science* e *PubMed* foram utilizadas cruzando os termos "*nanoparticles*" e "*bromelain*" para uma busca da literatura disponível sobre o uso de nanopartículas e bromelina na área farmacêutica (Capítulo III). Após a seleção das palavras-chave, foram encontrados 21 artigos (sem limitação do ano de publicação), dos quais 16 foram

revisados de acordo com a composição da nanopartícula, sendo que cinco artigos foram excluídos pois reportavam a purificação e quantificação da bromelina. Com base nesse trabalho de revisão, foi possível concluir que a imobilização ou encapsulação da bromelina tem se mostrado uma boa alternativa para o aumento de sua estabilidade, aumentando também suas potenciais aplicações. Além disso, a bromelina mostrou-se versátil, sendo utilizada também na funcionalização da superfície de nanopartículas, e servindo como agente redutor e de recobrimento na produção de nanopartículas.

Apesar do sucesso encontrado na combinação de bromelina e nanotecnologia, é possível notar que essa combinação ainda está no seu início e há espaço para pesquisas futuras. Dessa forma, o presente trabalho tem como principal objetivo a encapsulação de bromelina em nanopartículas de quitosana (Capítulos IV e V), a fim de aumentar sua estabilidade e modificar a liberação dessa enzima, visando sua utilização terapêutica no tratamento de feridas.

Em geral, os polissacarídeos, como a quitosana, são uma classe importante de materiais fisiológicos com propriedades atrativas como biocompatibilidade e biodegradabilidade, o que os torna uma escolha promissora para produzir nanopartículas para aprisionamento e entrega de bioativos (Sinha *et al.*, 2004; Lopes *et al.*, 2010). A quitosana é um polissacarídeo natural e abundantemente disponível, sendo derivada do exoesqueleto de crustáceos, insetos e fungos, além de apresentar características comuns a outros polissacarídeos, como biodegradabilidade, biocompatibilidade e bioadesão (Dash *et al.*, 2011). Condições de reação e extensão e fonte de quitina são fatores que podem ser modificados durante seu processo de desacetilação, e podem alterar as características finais da quitosana, como peso molecular, pKa e grau de desacetilação, alterando as propriedades físicas e químicas da quitosana (Wang *et al.*, 2011; Mohammed *et al.*, 2017).

Este polissacarídeo tem sido utilizado na liberação modificada de fármacos de várias classes terapêuticas, tais como antibióticos, anti-inflamatórios, anti-hipertensivos, peptídeos e proteínas (Bernkop-Schnürch, 2000; Florea *et al.*, 2006; Boonyo *et al.*, 2007; Pachioni-Vasconcelos *et al.*, 2016; Hasanifard *et al.*, 2017; Mohammed *et al.*, 2017). Além disso, a quitosana também tem sido estudada para o tratamento de feridas e queimaduras, com base em sua capacidade hemostática e seu efeito acelerado na reparação da ferida com melhor efeito estético final (Howling *et al.*, 2001; Argüelles, 2004; Dai *et al.*, 2011; Jayakumar *et al.*, 2011; Malmiri *et al.*, 2012; Felice *et al.*, 2015).

Diferentes métodos podem ser utilizados para a produção de nanopartículas de quitosana, como por exemplo, os métodos de emulsão, de formação de micelas reversas, de auto-montagem e de geleificação iônica (Sailaja *et al.*, 2011; Samyn *et al.*, 2018). Dentre os

quais, a geleificação iônica é frequentemente favorecida, por se tratar de um processo simples, controlável e pouco agressivo (O'callaghan and Kerry, 2016). Diferentes tipos de quitosana também podem ser utilizadas na produção das nanopartículas, conferindo diferentes propriedades a essas estruturas. Sendo assim, durante o período de estágio no exterior, quitosanas de diferentes fontes e com modificações foram testadas para incorporação de bromelina (Capítulo IV), a saber: quitosana de baixo peso molecular (LMW), quitosana oligossacarídeo de lactato (LAC), e quitosana de conchas de camarão (SHR).

Os três tipos de quitosana testados foram apropriados para a produção de nanopartículas pela técnica de geleificação iônica, como demostrado pela caracterização por *dynamic light scattering* (DLS) e espectroscopia no infravermelho com transformada de Fourier (FTIR). A incorporação de bromelina gerou, em todos os casos, uma diminuição no diâmetro médio das nanopartículas e da carga de superfície, com aumento da polidispersão. No entanto, a bromelina foi encapsulada com sucesso, com eficiência de encapsulação maior do 84% em relação a concentração de proteínas e maior do que 79% em relação a atividade enzimática. Os melhores resultados em termos de distribuição de tamanho das nanopartículas e de eficiência de encapsulação foram obtidos com a quitosana de baixo peso molecular.

A técnica de geleificação com tripolifosfato de sódio (TPP) tem sido bastante utilizada para a produção de nanopartículas e muitos autores reportam diferentes condições para essa produção (Shu and Zhu, 2000; 2002; Servat-Medina *et al.*, 2015; Goycoolea *et al.*, 2016; Severino *et al.*, 2016; Teimouri *et al.*, 2018). Sendo assim, um planejamento experimental 2^2 com triplicata do ponto central foi realizado. Neste planejamento, a relação de massa TPP/quitosana (10, 20 e 30%, m/m) e a velocidade de agitação (300, 550 e 750 rpm) foram utilizadas como *inputs*, enquanto que os *outputs* foram tamanho médio de partícula, índice de polidispersidade (PDI) e potencial zeta, determinados no equipamento ZetaSizer NanoZS (Malvern, Reino Unido).

Os *outputs* foram estatisticamente analisadas por diagrama de pareto e nenhum *input* interferiu significativamente no tamanho das partículas, polidispersão ou potencial zeta. Deste modo, a condição escolhida para o estudo da incorporação da bromelina foi: relação de massa TPP/quitosana de 30% e velocidade de agitação 350 rpm.

Assim, foram produzidas nanopartículas de quitosana-bromelina com a adição de 1 mL de solução de bromelina comercialmente disponível (10 mg/mL em água, filtrada em 0,22 µm), com aproximadamente 118,9 nm de diâmetro (Z-Ave), índice de polidispersão de 0,260 e potencial zeta de 21,1 mV. Nanopartículas de quitosana foram produzidas com a adição de 1 mL de água (filtrada em 0,22 µm) para comparação e como controle quando necessário. As

nanopartículas sem bromelaina apresentaram aproximadamente 254,5 nm de diâmetro, índice de polidispersão de 0,222 e potencial zeta +32,7 mV. A eficiência de encapsulação da bromelina foi determinada com base na diferença na concentração de proteínas e atividade enzimática da solução inicial de bromelina e do filtrado resultante da centrifugação das nanopartículas em dispositivos de ultrafiltração. Foram encontrados aproximadamente 87% e 81% de eficiência de encapsulação em termos de concentração de proteínas e atividade enzimática, respectivamente.

Além da técnica de *Dynamic Light Scattering* (DLS), as nanopartículas produzidas foram caracterizadas por *Nanoparticle Tracking Analysis* (NTA) e Microscopia Eletrônica de Varredura (MEV). Estudos tem reportado que a geleificação iônica com TPP pode levar a produção de nanopartículas de hidrogel de quitosana, que são materiais que possuem, ao mesmo tempo, características de hidrogéis e nanopartículas (Hamidi *et al.*, 2008; Pachioni-Vasconcelos *et al.*, 2016). As observações das imagens de MEV levaram a hipótese de que nanopartículas de hidrogel foram formadas neste estudo. Vale ressaltar que ainda se faz necessária a caracterização da amostra por Microscopia Eletrônica de Transmissão (TEM), que é a técnica de microscopia mais comumente reportada para a caracterização de nanopartículas, e sua caracterização química, através das técnicas de *Differential Scanning Calorimetry* (DSC) e de Fourier-*transform infrared spectroscopy* (FTIR).

O perfil de liberação *in vitro* da bromelina a partir das nanopartículas de quitosana-bromelina foi avaliado por 48 horas em tampão PBS pH 7.4 e em água. Uma liberação inicial em *burst* foi observada, atingindo 42.2% em água nas primeiras 2 horas, e 31.4% em tampão nas primeiras 6 horas. Após esse tempo inicial, foi possível observar uma queda na quantidade de proteína liberada, que não foi reportada por outros autores (Bhatnagar *et al.*, 2015; Bhatnagar *et al.*, 2016). Essa queda pode ser devido a limitações do método utilizado, como por exemplo agregação da bromelina liberada, o que não permitiu sua filtração nos dispositivos de ultracentrifugação. Outra possibilidade é a não-quantificação pelo método empregado, uma vez que seu limite de quantificação não permitiu detecções menores que 15%.

O aumento da estabilidade da bromelina através de sua nanoencapsulação foi avaliado por estudo de estabilidade de acordo com os parâmetros exigidos pela Agência Nacional de Vigilância Sanitária (ANVISA) (Anvisa, 2005) com as amostras de nanopartículas líquidas. Para isso, um novo lote de nanopartículas foi produzido seguindo os mesmos procedimentos, mas aumentando-se 20x o volume de produção. As nanopartículas produzidas apresentaram diâmetro médio e potencial zeta um pouco menor do que aqueles inicialmente encontrados, com

um aumento da polidispersão, o que pode ser devido ao aumento do volume sem alteração de outros parâmetros de produção, como por exemplo, a velocidade de agitação.

Durante o estudo de estabilidade preliminar, as amostras não apresentaram alterações macroscópicas e de pH, seguindo portanto, para o estudo de estabilidade acelerada. Nesse estudo acelerado, após 7 dias as nanopartículas de quitosana-bromelina apresentaram aumento de tamanho significativo, inclusive gerando erros de avaliação por DLS. Por outro lado, as nanopartículas de quitosana apresentaram significativa diminuição de diâmetro, mas ainda foi possível caracterizá-las por DLS. A concentração de proteínas e a atividade enzimática também foram monitoradas durante o estudo de estabilidade diretamente nas amostras de nanopartículas, e foram encontrados uma instabilidade com relação a concentração de proteínas e uma diminuição da atividade enzimática. Deste modo, as nanopartículas de quitosana-bromelina foram consideradas instáveis quando armazenadas em meio líquido, e foram submetidas a um estudo para sua liofilização.

Apesar de ser considerado um processo leve para a concentração ou secagem de produtos biologicamente ativos, a liofilização envolve duas condições desnaturantes para proteínas e/ou enzimas, o congelamento e a secagem (Roy and Gupta, 2004), e uma condição que pode induzir estresse para as nanopartículas, a secagem (Fonte *et al.*, 2016). Essas condições de estresse tanto para proteínas como para as nanopartículas podem ser contornadas através do uso de excipientes com propriedades crio- e lioprotetoras (Arakawa *et al.*, 2001; Roy and Gupta, 2004; Fonte *et al.*, 2016). Trealose, maltose e glicina foram escolhidos como potenciais protetores a serem utilizados com base em resultados previamente obtidos sobre a temperatura de colapso de excipientes (Geraldes, 2017).

A influência dos lioprotetores na temperatura de colapso das nanopartículas de quitosana-bromelina foi determinada pela observação microscópica direta do colapso, e os valores encontrados foram -56 °C, -49.3 °C, -34.8 °C e -28 °C para as nanopartículas de quitosana-bromelina e as nanopartículas com trealose, maltose e glicina, respectivamente. Durante a liofilização, a secagem primária deve acontecer abaixo da temperatura de colapso a fim de evitar o colapso do produto e a perda de sua estrutura macroscópica (Pikal and Shah, 1990; Abdelwahed *et al.*, 2006; Fonte *et al.*, 2016). De modo geral, processos de liofilização abaixo de -40 °C não são realizados, uma vez que o ciclo de secagem se torna mais lento e mais caro. Por isso, maltose e glicina foram selecionadas lioprotetores para o processo de liofilização.

O produto liofilizado obtido apresentou bom aspecto, sem encolhimento e curto tempo de ressuspensão para todas as amostras com lioprotetores. Os pós liofilizados foram analisados por análise termogravimétrica para determinação da umidade residual, apresentando 4,1% e

3,2% de perda de peso para as formulações com 3 e 5% (m/v) de glicina, respectivamente, e 5,6% e 3,8% para as formulações com maltose a 3 e 5% (m/v). Idealmente, a umidade residual deve ser inferior a 2% (Abdelwahed *et al.*, 2006; Sylvester *et al.*, 2018), o que não foi observado em nenhuma formulação.

Um desenho experimental 2^3 foi conduzido para avaliar os efeitos do tipo (maltose ou glicina) e da concentração (3% ou 5%, m/v) de lioprotetor utilizado, bem como os efeitos antes e após o processo de liofilização. O tipo de lioprotetor interferiu significativamente em quase todas as respostas analisadas, excluindo potencial zeta e eficiência de encapsulação em termos de atividade enzimática. De modo geral, o uso de glicina aumenta o diâmetro médio das nanopartículas, mas diminui o índice de polidispersão, indicando uma distribuição de tamanho mais restrita. A concentração dos lioprotetores também se mostrou como um fator significativo para o índice de polidispersão, sendo que menores concentrações (3%, m/v) diminuíram esse parâmetro.

Todas as respostas analisadas foram significativamente alteradas antes e após a liofilização, sendo que após a liofilização o diâmetro médio das nanopartículas aumentou, aumentando sua polidispersão e eficiência de encapsulação em termos de atividade enzimática. Além disso, o potencial zeta diminuiu, o que pode afetar a estabilidade das nanopartículas, e por isso um estudo de estabilidade da forma liofilizada deve ser posteriormente conduzido. Considerando todos os efeitos, formulações com 3% (m/v) de glicina ou maltose parecem promissoras e futuros estudos de estabilidade da forma liofilizada são necessários.

Além das propriedades previamente aqui mencionadas, a bromelina tem sido estudada com relação a sua atividade antioxidante (Manosroi *et al.*, 2014; Ataide *et al.*, 2017), atividade antiproliferativa (Chobotova *et al.*, 2010; Amini *et al.*, 2013; Bhatnagar *et al.*, 2014; Romano *et al.*, 2014; Gani *et al.*, 2015; Bhatnagar *et al.*, 2016; Oliveira *et al.*, 2017), e a melhoria na cicatrização de feridas (Bromelain. Monograph, 2010; De Lencastre Novaes *et al.*, 2016). Durante este trabalho as atividades antioxidante e antiproliferativa da bromelina livre e encapsulada, bem como sua atividade na retração de feridas *in vitro* foram investigadas (Capítulo VI).

Neste trabalho, dois métodos amplamente utilizados foram escolhidos para a determinação da atividade antioxidante *in vitro* da bromelina livre e encapsulada: DPPH e ABTS (Nile *et al.*, 2012). Conforme reportado anteriormente (Manosroi *et al.*, 2014; Ataide *et al.*, 2017), a atividade antioxidante da bromelina livre contra o radical DPPH foi dependente do tempo de reação e da concentração de bromelina utilizada, e esse mesmo comportamento foi observado para a bromelina nanoencapsulada, porém com uma menor porcentagem de inibição.

Inicialmente a bromelina não apresentou significativa atividade antioxidante no teste de ABTS, independente de sua forma (livre ou encapsulada). As amostras de ambos os testes foram então armazenadas ao abrigo da luz e fechadas por 24 horas, quando nova leitura espectrofotométrica foi realizada. Após 24 horas, a atividade nos testes de DPPH e ABTS havia aumentado tanto para a bromelina livre como para a nanopartícula de quitosana-bromelina.

Para a avaliação da atividade antiproliferativa, oito linhagens celulares tumorais e uma linhagem não-tumoral foram utilizadas, e a proliferação celular foi determinada pela quantificação de proteínas com sulforodamina B em 540 nm (Monks *et al.*, 1991; Bachiega *et al.*, 2016; Nunes *et al.*, 2017). Em ambos os casos, livre ou encapsulada, a bromelina não teve nenhum efeito nos queratinócitos, sugerindo segurança em seu uso tópico. Ainda neste ensaio, após 48 horas de exposição ao tratamento, a solução de bromelina inibiu o crescimento de 6 linhagens celulares tumorais (glioma, mama, próstata, ovário, adenocarcinoma de cólon e leucemia mielóide crônica), enquanto que a amostra de nanopartículas de quitosana-bromelina inibiu apenas o crescimento das células de leucemia mielóide crônica. Foi possível notar um aumento da GI_{50} (concentração que inibe 50% do crescimento celular) na linhagem de leucemia, ao comparar a bromelina encapsulada (GI_{50} = 204.4 µg/mL) com a livre (GI_{50} = 60.7 µg/mL). Outra observação importante foi que a bromelina livre foi capaz de inibir o crescimento das células de glioma (GI_{50} = 44.9 µg/mL), enquanto que as nanopartículas de quitosana-bromelina não apresentaram esse efeito.

Esses resultados, tanto de atividade antiproliferativa como de atividade antioxidante, apontam para um possível modificação na liberação da bromelina após a encapsulação, o que não foi confirmado pelo estudo de liberação *in vitro*. Com isso em mente, um segundo ensaio de atividade antiproliferativa *in vitro* foi realizado, utilizando uma linhagem tumoral e uma não-tumoral (glioma e queratinócitos, respectivamente), para avaliar o efeito do aumento do tempo de exposição para 144 horas (6 dias). Com o aumento do tempo de exposição, as duas formas da bromelina, livre e encapsulada, inibiram totalmente o crescimento das células de glioma, apresentando a mesma TGI (concentração que inibe totalmente o crescimento celular). No segundo ensaio, foi possível observar que a bromelina livre inibiu o crescimento dos queratinócitos (TGI = 119.33 µg/mL), porém após a nanoencapsulação esse efeito não foi mais observado.

Durante o ensaio de *scratch in vitro* utilizando células HaCat (queratinócitos), a bromelina apresentou menor retração da área da ferida quando comparada com as outras amostras. Além disso, as células tratadas com bromelina livre apresentaram indício de morte celular, como a presença de grânulos e espaços sem confluência. Aichele *et al.* (2013)

demonstrou que a bromelina atenuou moderadamente a proliferação de células endoteliais e de fibroblastos, através da condução dessas células ao estado de repouso do ciclo celular. Estes resultados podem ser indicativos de que a bromelina atue na cicatrização não pela proliferação ou migração de queratinócitos, mas sim por sua ação de debridamento, removendo restos celulares e tecidos necróticos *in vivo*, como o sugerido por outros autores (Singer *et al.*, 2010; Wu *et al.*, 2012).

Já as nanopartículas de quitosana e de quitosana-bromelina apresentaram o mesmo perfil durante o ensaio de *scratch*, promovendo 46% de retração da ferida após 9 horas, e atingindo 94% e 97% de retração, respectivamente, após 24 horas. Estudos anteriores demonstraram a ação da quitosana como promotora da cicatrização (Dai *et al.*, 2011; Felice *et al.*, 2015), indicando inclusive que elevados graus de desacetilação da quitosana estão associados a maiores estímulos a proliferação de fibroblastos (Howling *et al.*, 2001). Com base nos resultados obtidos é possível concluir que a nanoencapsulação da bromelina aumentou sua atividade na retração da ferida quando comparado a bromelina em sua forma livre.

Sendo assim, com base nos resultados observados na parte prática desse trabalho, pode-se concluir que é possível produzir nanopartículas de quitosana-bromelina através do método de geleificação iônica, com elevada eficiência de encapsulação. No entanto, as nanopartículas produzidas não são estáveis por longo período se armazenadas na forma líquida, e faz-se necessária sua liofilização a fim de efetivamente aumentar sua estabilidade. Após o processo de nanoencapsulação, a bromelina manteve sua atividade antioxidante e antiproliferativa, no entanto foram necessários maior tempo ou maior concentração para o mesmo efeito ser observado, o que sugere uma liberação modificada da bromelina. Além disso, a encapsulação da bromelina foi responsável por um aumento da retração da ferida no ensaio de *scratch* com queratinócitos.

5. CONCLUSÕES

Este trabalho apresenta os resultados do estudo de encapsulação da bromelina em nanopartículas de quitosana para aumento de sua estabilidade, visando com isso a ampliação das aplicações farmacêuticas e industriais desta enzima. As nanopartículas de quitosana-bromelina foram produzidas pela técnica de geleificação iônica e caracterizadas pelas técnicas de *dynamic light scattering*, *nanoparticles tracking analysis* e microscopia eletrônica de varredura, apresentando elevada e desejada eficiência de encapsulação. No entanto, as nanopartículas não se mostraram estáveis na forma líquida, sendo submetidas ao processo de liofilização. O produto liofilizado, com incorporação de glicina ou maltose como lioprotetores, apresentou curto tempo de ressuspensão, preservação do tamanho das nanopartículas e aumento da taxa de encapsulação quando comparado a forma líquida.

A bromelina em sua forma livre ou encapsulada não apresentou atividade antiproliferativa em queratinócitos, o que sugere segurança em seu uso tópico. Tanto na forma livre como na forma encapsulada, a bromelina apresentou atividade antioxidante e antiproliferativa. Essas atividades, no entanto, foram dependentes de maior concentração ou maior tempo de exposição para a bromelina encapsulada do que para a enzima livre, o que enfatiza seu aprisionamento nas nanopartículas de quitosana. As nanopartículas de quitosana-bromelina também aumentou efetivamente a retração de ferida no ensaio de *scratch in vitro* quando comparada a forma livre da bromelina. Sendo assim, as nanopartículas de quitosana-bromelina liofilizadas podem efetivamente melhorar a estabilidade da bromelina quando comparadas às formas líquidas, podendo levar a uma modificação de sua cinética de liberação, permitindo outras aplicações *in vivo* como um pó seco para administração oral/tópica ou como matéria-prima para outras formas de dosagem.

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7. APÊNDICES

Estes dados serão apresentados na forma de apêndice pois foram utilizados exclusivamente para que pudessemos garantir a realização dos experimentos. Sendo assim, não haverá redação de artigo sobre ele, mas são dados relevantes para a compreensão do trabalho como um todo.

7.1. Avaliação da estabilidade da bromelina na presença de polímeros e sais

Para os estudos de estabilidade, foram preparadas em tubos de ensaio soluções de bromelina padrão 10 mg/mL, quitosana 2,5mg/mL em ácido acético 1% (v/v), TPP 0,5 mg/mL em água destilada, e ácido acético 1% (v/v). Durante 7h, foram estudadas em triplicata a estabilidades de 4 soluções: 12 mL de bromelina sozinha, 2 mL de bromelina com 8mL de quitosana, 2 mL de bromelina com 12 mL de TPP, e 2,5 mL de bromelina com 10 mL ácido acético. As proporções foram determinadas com base no método que foi posteriormente utilizado para produzir as nanopartículas. As soluções foram submetidas a 3 condições: temperatura ambiente, geladeira e agitação. No tempo 0h e a cada hora subsequente do estudo, foram retiradas alíquotas a fim de analisar a atividade enzimática e a concentração de proteínas totais.

Para determinar a atividade enzimática da bromelina, foi utilizado o método de azocaseína (Sarath et al., 1989; Coelho et al., 2016). De forma resumida, o método propõe a clivagem da azocaseína pela bromelina a 37 °C por 10 minutos, até que a reação seja interrompida pela adição de ácido tricloroacético. A solução foi centrifugada, e uma alíquota do sobrenadante foi separada realizar a leitura a 440nm em espectrofotômetro (Genesys 10S UV-Vis, Thermo Scientific, Suécia). A atividade enzimática é calculada em unidades de atividade (U/mL), e por definição, uma unidade é a quantidade que causa aumento de uma unidade na absorbância de 1 mL de amostra em 60 minutos.

A determinação de proteínas totais foi realizada com o método de Bradford (1976). De modo geral, para a leitura do teste em cubetas, 20 μL de amostra e 1000 μL do reagente de Bradford foram adicionados em eppendorf de 2mL. A mistura foi agitada por 30 segundos em vórtex, incubada a 25 °C por 5 minutos, e a leitura foi feita em espectrofotômetro (Genesys 10S UV-Vis, Thermo Scientific, Suécia) a 595nm. Para a medida por espectrofotometria em leitor de microplaca (Multiscan GO, Thermo Scientific, Suécia), o procedimento foi o mesmo, com a diferença de que foram adicionados 5μL de amostra e 250μL do reagente de Bradford. Em

ambos os casos, a albumina foi utilizada para a elaboração da curva de calibração dos equipamentos (Figura 1).

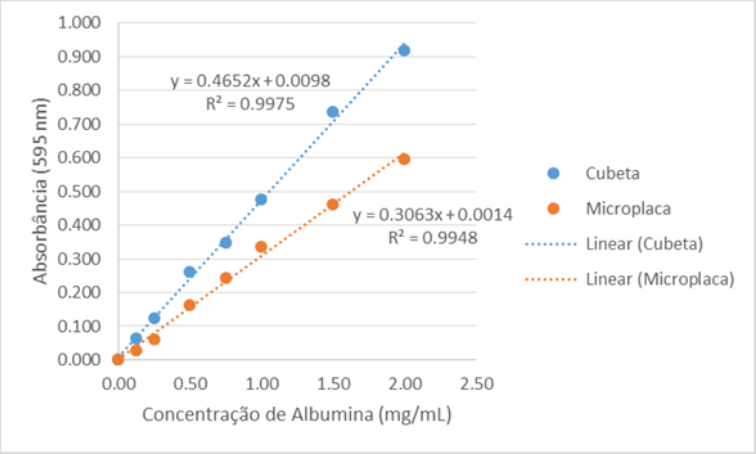

Figura 1. Curva de calibração do ensaio de Bradford com albumina em cubeta e em microplaca.

Para a solução de bromelina + quitosana também foi feito o teste de determinação da concentração de quitosana, utilizando uma adaptação do método descrito por Muzzarelli (1998). Primeiramente, uma solução tampão glicina foi preparada dissolvendo-se 1,87 g de glicina e 1,46 g de cloreto de sódio em 250 mL de água destilada. Alíquotas de 81 mL da solução foram coletadas e diluídas em 100 mL com HCl 0,1 M com pH 3,2. A solução corante foi feita com o reagente Cibacron Brilliant Red 3B-A (também conhecido como Reactive Red 4) obtido da MPBiomedicals (Califórnia, EUA), através da dissolução de 15 mg do reagente em 100 mL de água deionizada. Alíquotas de 5 mL da solução de corante foram coletadas e dilúidas para 100 mL em tampão de cloridrato de glicina 0,1 M com pH 3,2. A concentração final do corante foi de 0,075 g/L.

De modo geral, o procedimento descrito por Muzzarelli (1998), adiciona 300 µL de amostra e 3 mL do reagente Reactive Red 4 em tubo de ensaio, realizando a leitura de 1 mL da solução resultante em espectrofotômetro a 575 nm (Genesys 10S UV-Vis, Thermo Scientific, Suécia). Neste projeto, o método foi adaptado para ser realizado em microplaca, sendo assim misturou-se 30 µL de amostra e 300 µL de solução corante Em ambos os casos, tampão glicina foi usado como branco do aparelho. Para obter a curva de calibração, foi preparada uma solução estoque de quitosana 2 mg/mL em ácido acético 1% (v/v). Essa solução foi diluída em tampão glicina (0,1 M, pH 3,2) para se obter as concentrações desejadas de quitosana (25, 50, 75, 100, 150, 200, 250, 300, e 400 µg/mL).

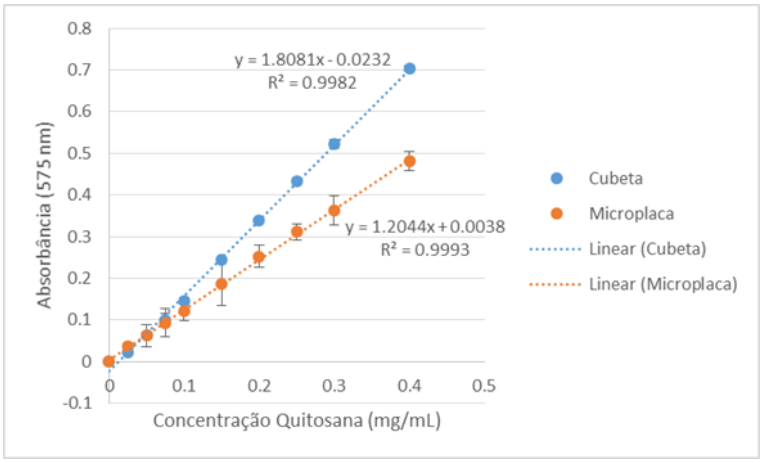

Figura 2. Curva de calibração do ensaio de concentração de quitosana em cubeta e microplaca.

No tempo 0, logo após ser misturada com TPP e ácido acético, não é observada uma variação significativa na concentração de proteínas totais, porém há uma redução significativa da atividade enzimática da bromelina, o que pode ser atribuído a uma desconformação de sua estrutura. Como pode ser visto nas figuras, a atividade enzimática sofre maior oscilação com o tempo do que a concentração de proteínas, o que também pode ser atribuído a alterações reversíveis na estrutura tridimensional da bromelina. A concentração de proteínas totais decai significativamente na solução de bromelina em água após 3 horas quando deixada em temperatura ambiente, e em 5 horas quando armazenada em geladeira ou sobre agitação. Apesar das reduções no conteúdo proteíco, todas as amostras mantiveram uma concentração mínima de aproximadamente 12 mg/mL, o que foi considerado adequado, permitindo a mistura da bromelina com o solução de TPP e ácido acético, necessária para o preparo das nanopartículas.

Quando misturada à quitosana, a bromelina não apresenta tantas variações em sua estabilidade em comparação às outras soluções até atingir 5h, quando é observado um aumento na atividade enzimática e na concentração de proteínas totais, o que também pode ser atribuido a alterações tridimensionais da bromelina.

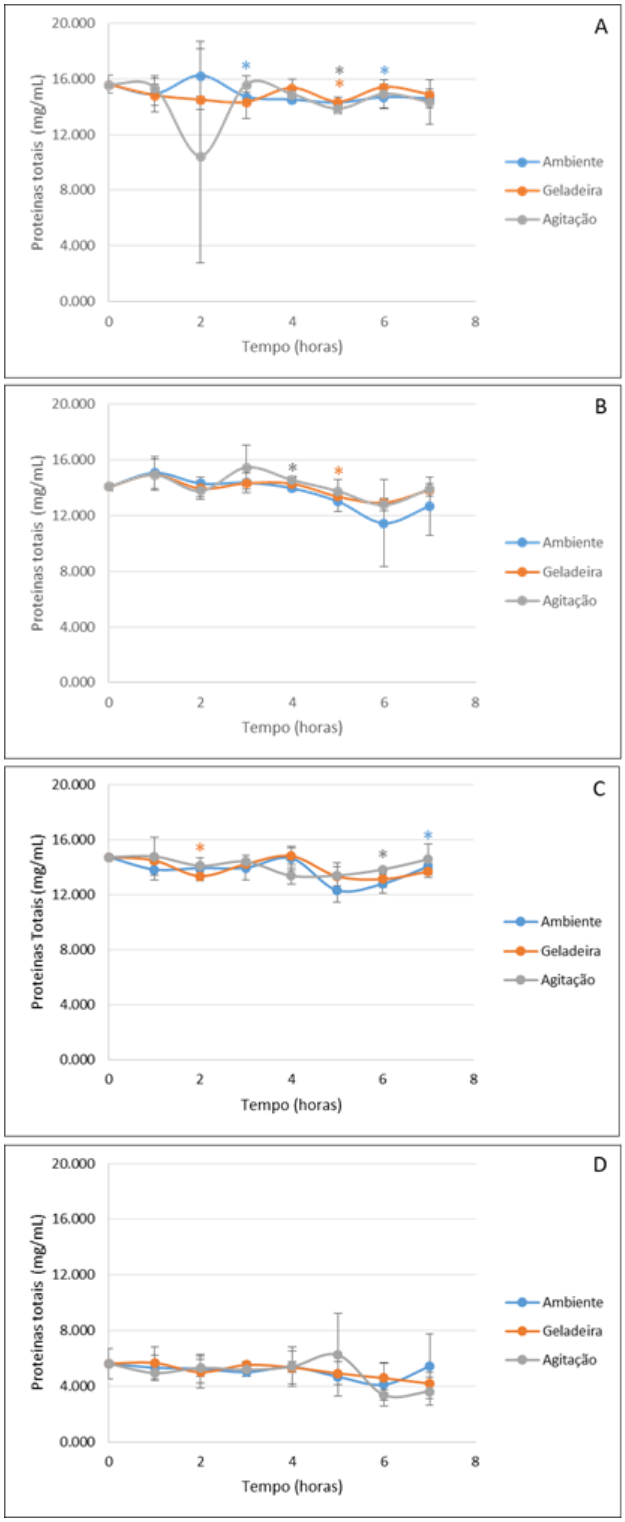

*Figura 3. Avaliação da concentração de proteínas totais da bromelina em água (A), solução de tripolifosfato de sódio (B), ácido acético (C) e quitosana (D), onde * sinaliza onde houve diferença estatisticamente significante ($p < 0.05$) em comparação ao tempo 0, seguindo a legenda de cores das condições estudadas.*

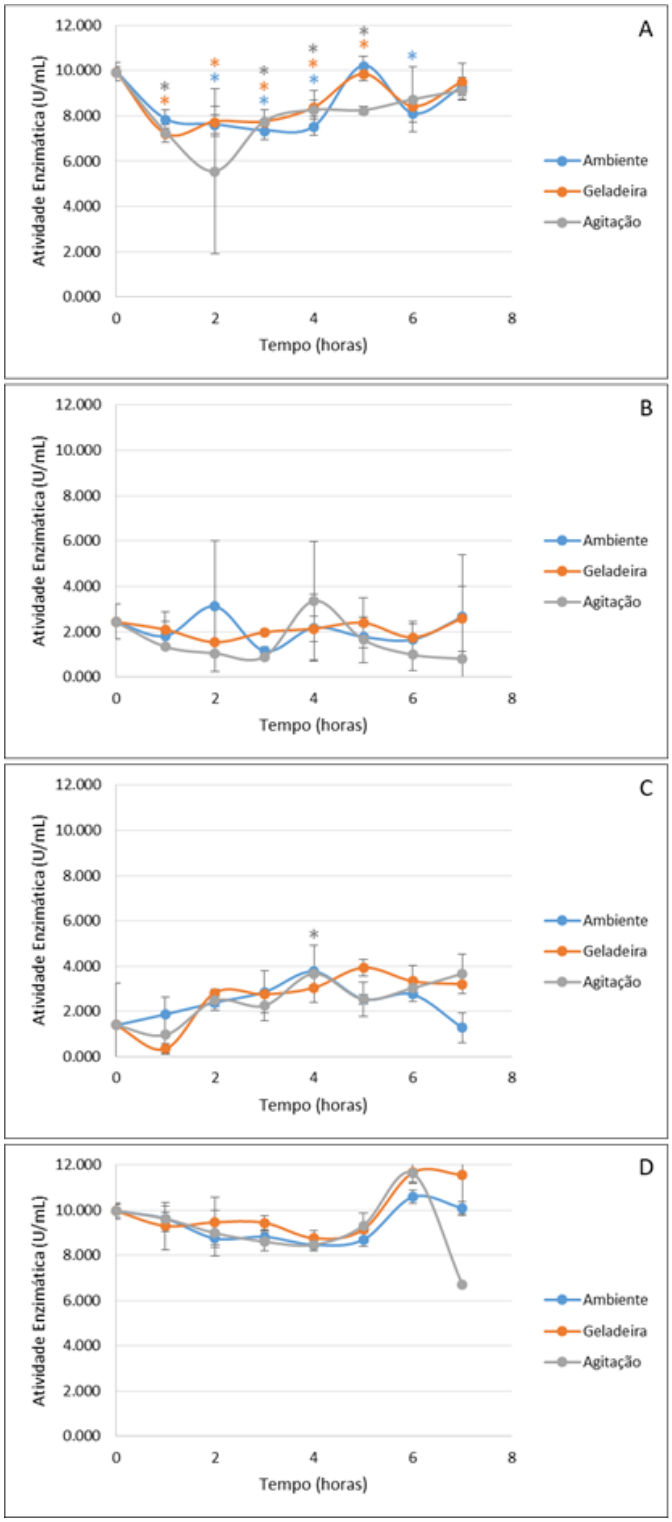

*Figura 4. Avaliação da atividade enzimática da bromelina em água (A), solução de tripolifosfato de sódio (B), ácido acético (C) e quitosana (D), onde * sinaliza onde houve diferença estatisticamente significante ($p < 0.05$) em comparação ao tempo 0.*

A solução de bromelina + quitosana também foi avaliada quanto a concentração de quitosana pelo método de Muzzarelli (1998) e o resultado está apresentado na Figura 5. A

concentração de quitosana mantem-se praticamente constante ao longo de todo o tempo de estudo, apresentando uma pequena diminuição a partir de 6 horas, que é mais pronunciada após 7 horas em temperatura ambiente. Sendo assim, pode-se dizer que a clivagem da quitosana pela bromelina foi pequena e praticamente não observada neste experimento. Lee et al. (2005), demonstraram a clivagem da quitosana pela bromelina em uma situação diferente deste experimento: 1% quitosana (m/v) e 7% bromelina (m/v), a 50 °C por 2 horas, ou seja, para a clivagem acontecer a concentração de bromelina deve ser maior do que a concentração de quitosana, o que não acontece nas condições de desenvolvimento deste projeto.

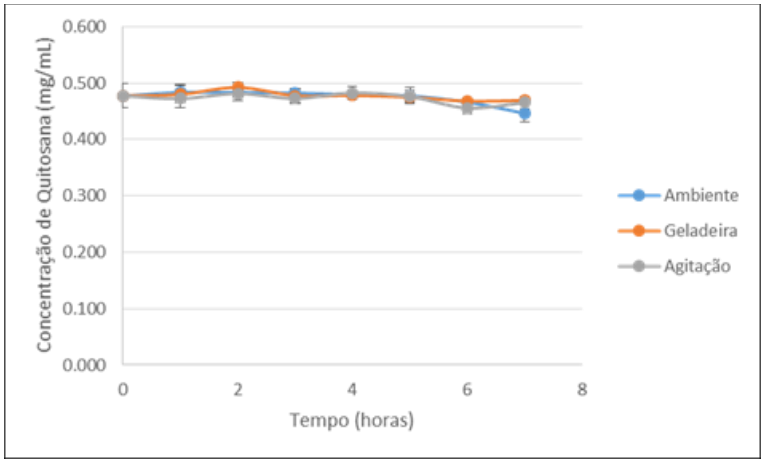

Figura 5. Determinação da concentração de quitosana.

7.2. Ensaio de micro-Bradford

Para a determinação de pequenas quantidades de proteína foi utilizado o teste de micro-Bradford de acordo com o protocolo estabelecido pela empresa Sigma-Aldrich. De modo geral, o mesmo volume de amostra e reagente de Bradford devem ser misturados e a absorbância lida em 595 nm. Essa metodologia foi adaptada para microplaca de 96 poços e então, 200 µL de amostra foram misturados com 200 µL de reagente. A curva de calibração (Figura 6) foi feita utilizando albumina de soro bovino como padrão. Foram determinados os limites de detecção e quantificação deste método, obtendo valores de 3,47 µg/mL e 10,52 µg/mL, respectivamente.

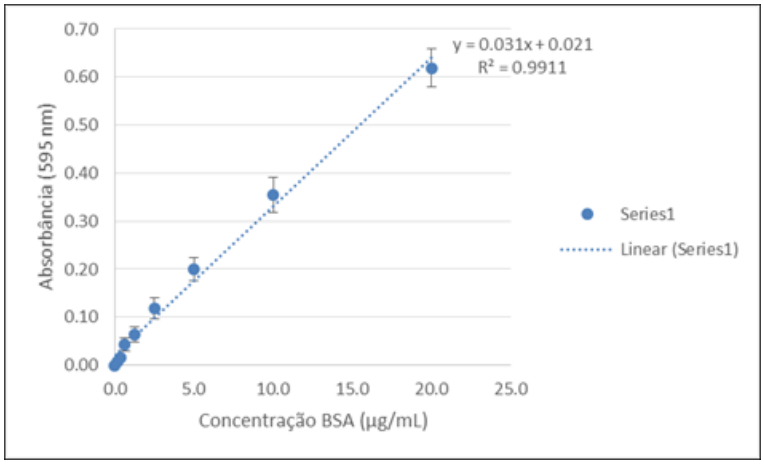

Figura 6. Curva de calibração do ensaio de micro-Bradford em microplaca.

7.3. Nanopartículas de quitosana: caracterização

Apesar de não constar no artigo apresentado no Capítulo V desta dissertação, as nanopartículas de quitosana, utilizadas como controle para validação dos ensaios realizados com as nanopartículas de quitosana-bromelina, foram caracterizadas por *dynamic light scattering* e *nanoparticles tracking analysis* (Tabela I).

Tabela 1. Caracterização físico-química das nanopartículas de quitosana.

		DLS	NTA
Diâmetro (nm)	**Z-ave**	120,5 ± 1,5	185,7 ± 14,9
	D_{10}	71,6 ± 2,2	108,5 ± 5,9
	D_{50}	126,0 ± 2,0	152,7 ± 7,1
	D_{90}	240,0 ± 11,7	273,9 ± 39,5
PDI		0.213 ± 0.014	-
Potencial Zeta (mV)		+23,5 ± 1,7	-
Concentração (partículas/mL)		-	(1.18 ± 0.06) x 10^{12}

**Resultados apresentados como média ± desvio padrão de três medidas. DLS = dynamic light scattering; NTA = nanoparticles tracking analysis; PDI = índice de polidispersão.*

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8. ANEXOS

8.1. Artigos Publicados

Advances in Colloid and Interface Science 254 (2018) 48–55

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Advances in Colloid and Interface Science

journal homepage: www.elsevier.com/locate/cis

Historical perspective

Bromelain-loaded nanoparticles: A comprehensive review of the state of the art

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ABSTRACT

Stem bromelain is a common available cysteine protease derived from pineapple (*Ananas comosus* L.). Bromelain finds widespread applications in several areas, such as medicine, health, food, and cosmetics, and its strong proteolytic activity supports its future application in many additional fields. However, most proteins and/or enzymes are fragile, leading to important considerations about increase storage and operational stability to enable their practical application. In this scenario, the use of nanoparticles to deliver proteins is increasing exponentially, given that these systems are capable of enhance active's stability, solubility and permeability, and decrease toxicity. In the pharmaceutical nanotechnology field, bromelain has played different roles and thus this paper aims to review the available literature for the use of nanoparticles and bromelain.

Contents

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1. Introduction

Bromelain is a crude extract derived from pineapple plant (*Ananas comosus* L.) and contains mixture of proteolytic enzymes and non-enzymatic substances [1]. It can be found in several parts of the pineapple plant, including its stem, fruit, leaves and peel [2,3]; only the stem and fruit, however, produce high amounts of bromelain [4,5]. Stem

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REVIEW

WILEY

Natural actives for wound healing: A review

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Nature has been a source of medicinal treatments for thousands of years, with the use of plants as prototypes for drug development and for the extraction of active compounds. Skin injuries occur regularly in everyday life, and the human skin has the ability to promote repair spontaneously under healthy conditions. However, some intrinsic and external factors may interfere with skins' natural ability, leading to nonhealing lesions and chronic wounds, which directly affect health and quality of life. Thus, attention should be given to this health problem, using an appropriated management when necessary. In this scenario, phytotherapy may be an option for cutaneous wound treatment, although further high-quality studies are needed to firmly establish the clinical efficacy of plants. This article reviews traditionally used natural actives for wound healing, highlighting their characteristics and mode of action.

KEYWORDS
natural actives, topical actives, wound healing

1 | INTRODUCTION

The largest organ of the human body is the skin, accounting for approximately 15% of total body weight in adults (Mulholland, Dunne, & McCarthy, 2017; Tobin, 2006). It acts as a protective barrier against the external environment and helps to prevent dehydration (Mulholland et al., 2017), which highlights the importance to maintain its integrity. Reconstructing functional skin after a wound remains a challenge due to the complexity of healing, involving orchestrated cell-signalling events and biochemical cascades (Berthet, Gauthier, Lacroix, Verrier, & Monge, 2017).

After damage, human skin promotes spontaneous repair by a four-stage process that results in the formation of non-functional fibrotic tissue (Gurtner, Werner, Barrandon, & Longaker, 2008). Impairments at any one or more of these stages can lead to compromised healing (Mulholland et al., 2017), which can lead the wound to enter in a chronic state, affecting patients' health and quality of life (Abdelrahman & Newton, 2011; Pereira & Bártolo, 2016). Thus, wound management is important to avoid complications, comprising appropriated topical pharmacotherapy and dressings (Atiyeh, Costagliola, Hayek, & Dibo, 2007; Fraser, Cuttle, Kempf, & Kimble, 2004; Salas Campos, Fernandes Mansilla, & Martinez de la Chica, 2005), which make chronic wounds a major target for medical technological development (Gueldner, Zhang, Zechmann, & Bruce, 2017). Conventional therapies include the use of synthetic antibiotics and healing promoters (Hajská, Dragúňová, & Koller, 2017) and dressings that are used to protect dermal and epidermal tissues (Felgueiras & Amorim, 2017). However, these treatments and dressing are mostly inadequate for chronic wounds (Mulholland et al., 2017).

For thousands of years, nature has been a source of medicinal treatments, and plant-based systems continue to play an essential role in primary health care around the world, with natural compounds been used in skin wound care particularly due to their antiinflammatory, antimicrobial, and cell-stimulating properties (Pazyar, Yaghoobi, Rafiee, Mehrabian, & Feily, 2014; Pereira & Bártolo, 2016). Many plants and their extracts have been traditionally used due to their great potential for wounds management and treatment, with these agents inducing healing and tissue regeneration through multiple connected mechanisms (Maver, Maver, Stana Kleinschek, Smrke, &

8.2. Artigos Submetidos

Wound Healing Process and Synthetic Actives: a Review

Autores: Janaína Artem Ataide, Letícia Caramori Cefali, Lucas Militão, Beatriz Zanchetta, Eliana B. Souto, Laura Oliveira-Nascimento, Priscila Gava Mazzola

Revista: European Journal of Pharmaceutical Sciences

8.3. Prêmios

Prêmio de 3º Lugar de Apresentação Oral, II Workshop in Environmental Nanotechnology, realizado na Universidade de São Paulo campus Sorocaba. Sorocaba/SP. Trabalho: "Bromelain encapsulation in chitosan nanoparticles". 2016.

Menção honrosa na 10ª Semana de Pesquisa da Faculdade de Ciências Médicas da Unicamp, Campinas/SP. Trabalho: "Encapsulação de bromelina em nanopartículas de quitosana". 2017.

8.4. Trabalhos Apresentados em Conferências

ATAIDE, J.A., GÉRIOS, E.F., BISSACO, F.M., CEFALI, L.C., NASCIMENTO, L.O., MAZZOLA, P.G. Bromelain encapsulation in chitosan nanoparticles. Apresentação oral feita por Janaína Artem Ataide no II Workshop in Environmental Nanotechnology, realizado na Universidade Estadual de São Paulo, Campus Sorocaba, de 7 a 9 de dezembro de 2016.

ATAIDE, J.A., GÉRIOS, E.F., BISSACO, F.M., NASCIMENTO, L.O., MAZZOLA, P.G. Encapsulação de bromelina em nanopartículas de quitosana. Apresentação em poster feira por Janaína Artem Ataide na 10ª Semana de Pesquisa da Faculdade de Ciências Médicas da Unicamp, realizada em Campinas, 2017.

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feita por Janaína Artem Ataide no Trends in Natural Product Research, durante o congresso realizado em Villeneuve d'Ascq (Polytech Lille), de 28 de junho a 01 de julho de 2017.

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8.5. Declarações

8.5.1. Comitê de Ética

DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "**DESENVOLVIMENTO DE PARTÍCULAS POLIMÉRICAS COMO SISTEMAS CARREADORES DE PRODUTO BIOAT**IVO", desenvolvida no Programa de Pós-Graduação em Ciências Médicas da Faculdade de Ciências Médicas da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Data: 09/01/2018

8.5.2. Direitos Autorais

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