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TANNIC ACID-LOADED CHITOSAN MICROPARTICLES FOR THE TREATMENT OF DIABETIC WOUNDS

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by
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Resumo

A ferida diabética é considerada uma das complicações mais comuns de diabetes *mellitus* devido a um processo de cicatrização afetado por condições hiperglicémicas. Nos últimos anos, o número de diabéticos com feridas crónicas aumentou globalmente devido tanto à falta de medidas preventivas e de controlo, como ao desenvolvimento de bactérias resistentes a antibióticos. Desta forma, a descoberta de um tratamento eficiente tornou-se um desafio na área da saúde, onde novos curativos que incorporam agentes bioativos têm sido estudados para melhorar o processo de cicatrização. O potencial biológico dos polifenóis, bem como a sua natureza, permite-lhes ser uma alternativa eficiente em comparação aos antibióticos, dando resposta à necessidade de encontrar novas formas de tratamento de feridas crónicas. No entanto, compostos fenólicos como o ácido tânico (TA) podem apresentar algumas limitações quando direcionados para aplicações na ferida, como baixa estabilidade e consequente desempenho biológico limitado no local da ferida. Como forma de colmatar estas falhas e melhorar a libertação e o desempenho dos polifenóis, microssistemas têm sido desenvolvidos como sistemas de entrega. Como transportador de escala micrométrica, o quitosano (CS) tem demonstrado bons resultados na libertação gradual de diversos compostos, como polifenóis, devido às suas características biofuncionais excelentes. Desta forma, acredita-se que a microencapsulação tendo por base CS como material encapsulante permita a entrega e libertação controlada de TA, promovendo a sua biodisponibilidade.

O objetivo deste estudo foi desenvolver e caracterizar um curativo inovador em pó, composto por TA, CS e sericina de seda (SS), com propriedades antimicrobianas e antioxidantes para o tratamento de feridas diabéticas. O TA foi encapsulado em micropartículas de CS (CMTA) por secagem por atomização, e a sua capacidade antimicrobiana e antioxidante bem como o perfil de libertação foram estudados. Para atribuir propriedades hidratantes e regenerativas ao pó final, SS foi adicionada posteriormente.

Como resultado, obteve-se um rendimento satisfatório de 32% para o processo de secagem, mostrando ainda ótimos resultados para a eficiência de encapsulação (98,50%). O diâmetro obtido foi de 7,4 µm, CMTA exibiram uma morfologia esférica com concavidades na superfície externa, enquanto que micropartículas hidratadas apresentaram uma forma regular de superfície lisa com alguma tendência de aglomeração. A estabilidade térmica de CMTA foi comprovada e não foram encontradas ligações covalente entre TA e CS. Em relação às propriedades biológicas, TA encapsulado apresentou menores valores de atividade antioxidante, em comparação com TA livre em solução, devido ao seu sistema de encapsulação. No entanto, CMTA mostrou um ótimo desempenho na atividade antioxidante, para além de proteger o TA de degradação, proporcionar estabilidade biológica bem como uma libertação controlada (~0,8%). As micropartículas foram ainda bactericidas contra *Staphylococcus aureus* e *Staphylococcus aureus* resistente à meticilina. No entanto, apesar do seu potencial cicatrizante, a SS não foi um ganho para melhorar a atividade antioxidante ou antimicrobiana de CMTA. O trabalho permitiu o desenho experimental para o desenvolvimento e produção de CMTA. Acredita-se que as partículas CMTA têm potencial biológico para a entrega de TA em feridas complexas. No entanto, novos estudos devem ser realizados de modo a obter uma melhor caracterização de CMTA, como um sistema de libertação mais controlado e sustável.

Palavras-chave: Quitosano, ferida diabética, microencapsulação, sericina, ácido tânico, cicatrização

Abstract

Diabetic wound is considered one of the most common complications of patients with diabetes due to an impaired healing process affected by hyperglycemic conditions. In recent years, the number of diabetics with chronic wounds are globally increasing, due to lack of preventive and controlling measures as well as the development of resistant bacterial strains to antibiotics. Thus, an efficient treatment has become a health challenge, where new dressing materials incorporating bioactive agents have been studied to improve the healing process. The biological potential of polyphenols as well as their nature allows them to be an efficient alternative as compared antibiotics, responding to the urge to find new alternatives for chronic wound care. However, phenolic compounds like tannic acid (TA) may have some drawbacks, when targeting wound applications, such as low stability and consequent biological performance in the wound site. To overcome these limitations and improve the release and the performance of polyphenols, microsystems have been developed as a delivery system. As a microcarrier, chitosan (CS) has demonstrated great results in the gradual release of several drugs, such as polyphenols, due to its outstanding biofunctional properties. Therefore, microencapsulation based on CS is expected to provide a target delivery of TA as well as its controlled release and increased its bioavailability.

The aim of this study was to develop and characterize a new powdered wound dressing with antimicrobial and antioxidant properties to apply in chronic wounds, composed by silk sericin (SS), TA, and CS. Tannic acid was encapsulated in CS microparticles by spray drying, and its antimicrobial and antioxidant capacity as well as the profile release of TA were evaluated. To assign hydrating and regenerative properties of the final powder, SS was added.

As results, a satisfactory product yield of 32% were obtained for spray drying process, showing great results for encapsulation efficiency (98.50%). Diameters obtained were around 7.4 μm, exhibited a spherical morphology with concavities on the outer surface, while hydrated microparticles were regular shape and smooth surface with some agglomeration tendency. Thermal stability of CMTA was proven, and no covalent bonds were found between TA and CS. Regarding biological properties, TA encapsulated showed lower values of antioxidant activity, compared to TA free in solution due to its encapsulation system. Nevertheless, CMTA still have optimal antioxidant activity performance, plus protecting TA from degradation and providing biological stability, and controlled released (~0.8%). The microparticles were also bactericide against *Staphylococcus aureus ATCC* as well as methicillin resistance *Staphylococcus aureus*. However, although its greats potential for wound healing, SS was not a gain to improve the microparticles antioxidant potential or either antimicrobial of CMTA properties.

The work allowed an efficient microencapsulation process for CMTA design, which leads to believe that microparticles may have a great biological potential for TA wound delivery. However, further studies should be performed in order to get a better characterization of CMTA, and, to better understand and to obtain a more controlled and sustainable release system.

Keywords: Chitosan, diabetic wounds, microencapsulation, sericin, tannic acid, wound healing

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"A gratidão é a memória do coração"
Antístenes

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List of abreviations

ABTS	2,2-Azinobis (3-Ethyl-benzothiazoline-6-Sulforic acid)
AE	Association Efficiency
CFU	Colony-forming unit
CMTA	Tannic acid loaded chitosan microparticles
CMTA-SS	Tannic acid loaded chitosan microparticles with silk sericin
CS	Chitosan
DFI	Diabetic foot infection
DFU	Diabetic foot ulcer
DSC	Differential Scanning Calorimetry
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared
HPLC	High Performance Liquid Chromatography
LD50	Median lethal dose
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
PBS	Phosphate-buffered saline
ROS	Reactive Oxygen Species
rpm	Rotations per minute
SEM	Scanning electron microscope
SS	Silk sericin
TA	Tannic acid

CHAPTER 1

INTRODUCTION

“There is not a discovery in science, however revolutionary, however sparkling with insight, that does not arise out of what went before.”
Isaac Asimov

CHAPTER 1: INTRODUCTION

1.1. Diabetic wounds

Diabetes, one of the most prevalent chronic diseases, is characterized by ineffective insulin synthesis or resistance of human cells to insulin binding, creating hyperglycemic conditions in the human body (1). The prevalence of diabetes is increasing worldwide and are planned to continue to rise over the next years, being the greatest number of diabetics people with ages between 40 and 60 (2). In 2018 it was estimated that more than 422 million people worldwide suffered from diabetes and this is expected to rise to 642 million by 2040 (3, 4). World Health Organization reports that diabetes causes 1.6 million deaths in 2016, and, according to statistical numbers, it is believed that can become the 7th leading cause of death by 2030 (5). Therefore, diabetes became one of the major public health challenges, reason why is so important to develop new controlling strategies of this disease as well as inherent complications (4).

Diabetic wounds, mainly chronic and/or complex wounds, are considered one of the most common complications associated to diabetes mellitus (6). Its long healing process, that normally takes more than 12 weeks, approximately, may result in ulceration and serious infections, and, in the worst case, may lead to amputations like low extremity amputation, in case of diabetic foot ulcers (DFU) (6, 7). Diabetic foot ulcer is considered one of the major concerns of diabetics because it significantly compromises the quality of life of the patient. It represents a higher percentage of hospital admissions, which also results in increasing morbidity and mortality (8). Therefore, diabetes became an economic problem, as 2.5%-15% of yearly worldwide health budgets are consumed on diabetes *mellitus* and diabetic wounds represent much larger expenses (9). Moreover, hospital-based studies have shown that mortality rates among diabetic patients with DFUs are around twice higher than those in patients with diabetes who do not suffer from foot ulcers. Both types of diabetes (type 1 and type 2) are likely to develop DFUs (10). It is estimated that 19-34% of patients with diabetes are more prone to be affected by DFUs, which represents 9.1 million to 26.1 million people worldwide (2, 9, 11). However, this number is globally increasing due to a lack of preventive and controlling measures (6). Unfortunately, in some cases the only possible solution for DFUs is amputation (12). About 50-70% of all limb amputations worldwide (more than 1 million people annually) are caused by diabetic wounds (6, 7, 10). For this reason, it is of great importance to understand why it is so difficult to heal a diabetic wound, and find new alternatives for ulcer treatment or optimize strategies of wound healing process already existing (1, 7).

1.1.1 Impaired wound healing

Wound healing is a continued physiologic process that repairs and regenerates the damage tissue after a trauma (13). Normally, the healing process goes through four main basic phases including hemostasis, inflammatory, proliferative, and remodeling (Figure 1) (13, 14).

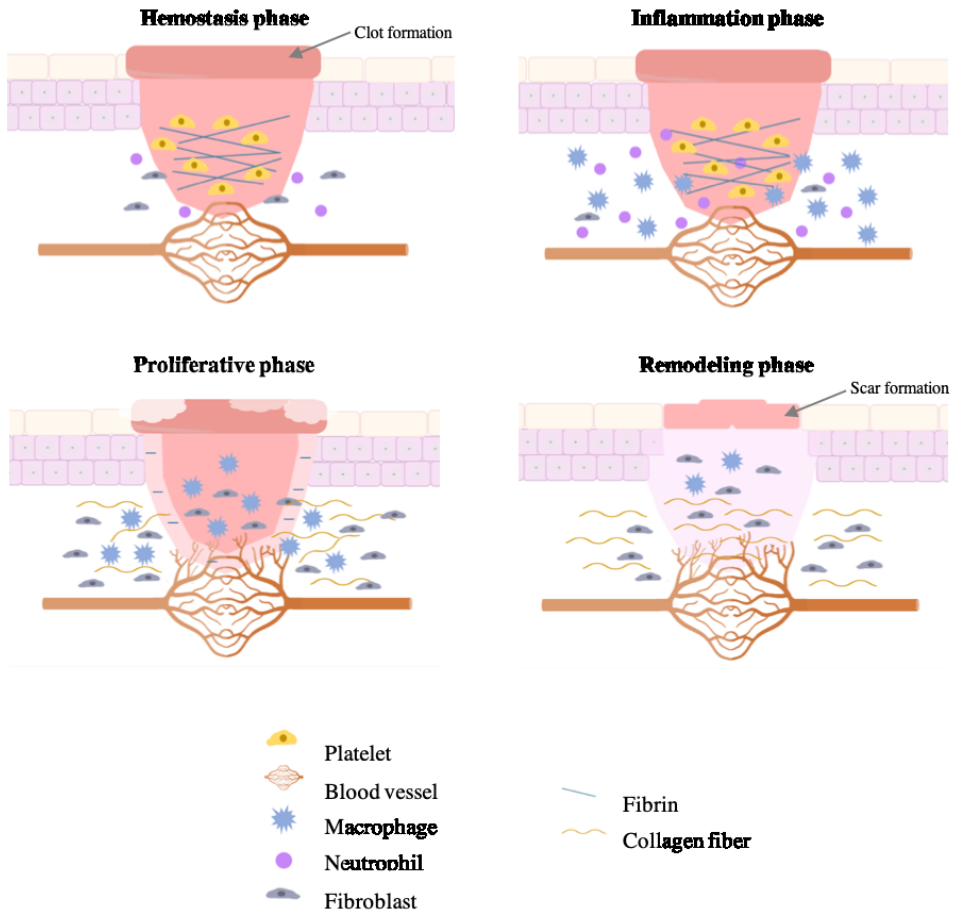

Figure 1 – Different phases of normal wound healing

The hemostasis phase is characterized by current platelet activation, which results in releasing chemokines and growth factors to form the clot (13, 15). After the coagulation is complete, platelet is reinforced with a fibrin network acting like a molecular binding agent to form a barrier against microorganisms as well as organize the matrix for cell migration (15, 16). In response to inflammatory signs, phagocytes (neutrophils and macrophages) migrate to the wound facilitating phagocytosis of bacteria and damage tissue (14, 17). Both cells are responsible for the production of cytokines and growth factors, that are necessary to an efficient wound healing process (6). Before the proliferative phase, macrophages are responsible to release cytokines and residual neutrophils by apoptosis, which allows the healing process to proceed. In response of growth factors, the wound closure is also characterized by the deposition of collagen fibers produced by the migration of fibroblasts to promote the production of extracellular matrix (13, 16). Additionally, macrophages are responsible for stimulating the formation of new blood vessels at the wound brim (angiogenesis) providing oxygen, nutrients and metabolite, and to rebuild the initial clot with new tissue formed by extracellular matrix (granulation tissue). Thus, granulation tissue creates the conditions needed to re-epithelialization (13, 14, 16). Finally, the remodeling phase is characterized by the resynthesize of the extracellular matrix, being necessary a balance between apoptosis of existing cells and production of new cells. This phase begins

when the collagen is remodeled from type III of the granulation tissue to type I, and becomes aligned in parallel bundles, which increase the tensile strength and the wound fully closes (14, 17).

However, diabetes is responsible for a significant delay on the repair of anatomical integrity of wounds due to an interruption of healing process in the inflammatory phase, where there are functional alterations in cells (6, 18). As a result of the dysfunctional healing, diabetic wounds are highly prone to develop infections. Diabetic wound infections will be addressed in more detail on the following sub-chapter. The disruption of this biologic process may occur by intrinsic and extrinsic factors. Extrinsic factors are attributed to wound infection and unnoticed and repetitive trauma, which coupled with intrinsic factors makes the inflammatory phase of diabetic wounds much more prolonged (18-20). Intrinsically, there is a progressive loss of peripheral nerve fibers caused by high glycemic levels, affecting the outer nerves of the limbs (6, 19). As a consequence of hyperglycemic conditions, vascularization becomes impaired by a decreased capillary size and basement membrane thickening, thus limiting the peripheral blood flow and migration of dermal and epidermal cells into the wound (6). A schematic representation of diabetic wound healing process is presented in Figure 2.

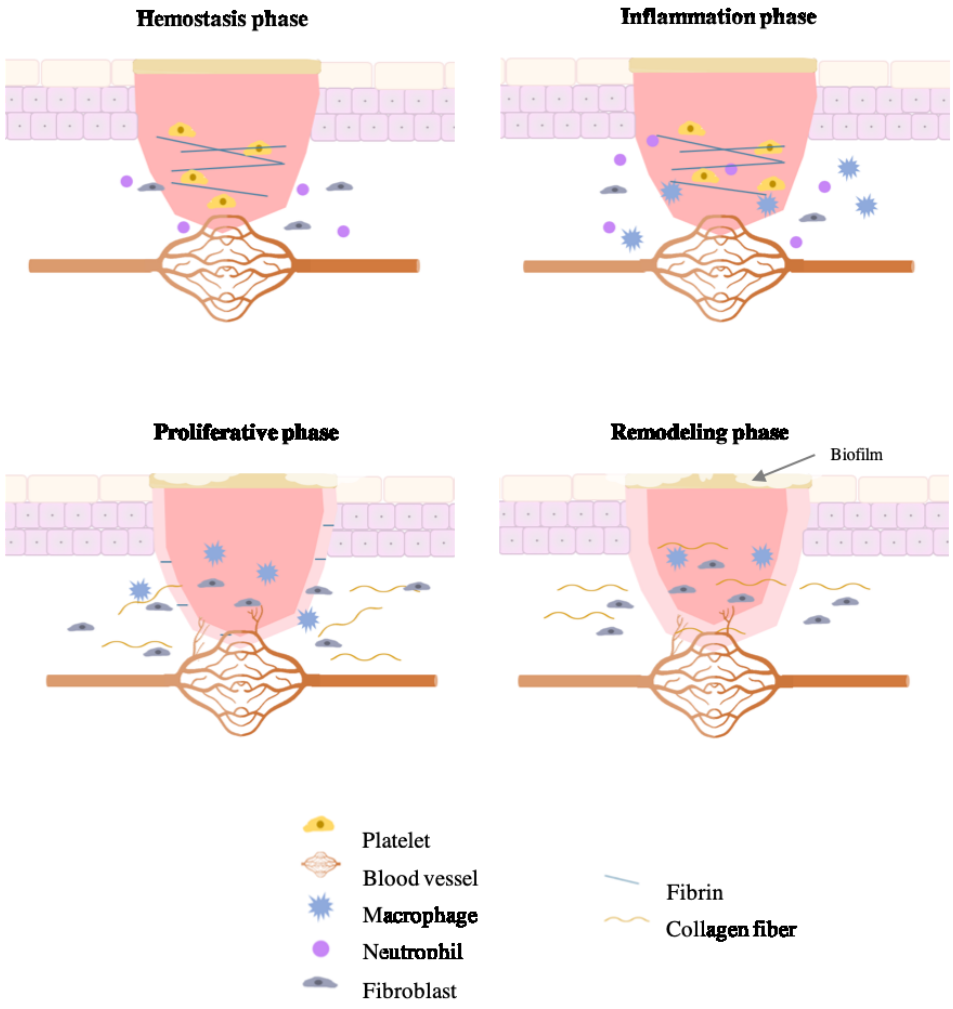

Figure 2 – Different phases of diabetic wound healing

The impaired wound healing of diabetic wounds is also characterized by a disarranged conversion of monocytes into macrophages that hamper the inflammatory phase, since the surrounding tissue is destructed. In addition, the function of macrophages to clear apoptotic neutrophils by phagocytosis is decreased, resulting in a persistent of neutrophils and, consequently, in a delay of healing process of diabetic wounds (18). This influx of neutrophils is responsible for the release of proteases, cytotoxic enzymes, as well as free radicals, such as hydrogen peroxide (H_2O_2) and superoxide (O_2), resulting in an overproduction of these compounds that becomes prejudicial to the diabetic wound when compared to the normal healing process (6, 15, 18). As a result, production of compounds of extracellular matrix or even growth factors can be affected, compromising the normal cell function (14). Relatively to fibroblasts, there is a decrease of their migration to the wound site, that contributing to a dysfunctional epithelialization as well as an impaired angiogenic response (6, 18). Along with these, there are also an impaired collagen accumulatio (21).

Moreover, there are still some controllable extrinsic factors that can also aggravate the diabetic wound healing process. As an example, poor nutrition can be responsible for reducing the proliferation of fibroblasts and the synthesis of collagen. Cigarette smoking, alcohol consumption, depression and anxiety and even disturbed sleeping are also factors that contribute to the development of diabetic wounds (21-23). All these intrinsic and extrinsic factors associated to the impaired healing of diabetic wounds negatively affect extracellular matrix deposition, host immune response, as well as the access to antibiotics to the wound site, in case of infections (20, 24).

1.1.2 Prominent infection and strategies for its control

Because diabetic wounds are chronic, prominent infections are often associated (20, 24). Wound infection is characterized by the colonization of microorganisms, usually bacteria, capable of developing colonies in the wound site. Consequently, while the healing process is delayed, the wound becomes increasingly impenetrable by the antimicrobial agents (9). Another consequence associated with wound infection is the oxidative stress, which also retards the physiological healing process (25). The typical oxidative stress of chronic wounds is detailed on the following section. Coupled with the impaired immunological system of diabetics, wound infections management becomes serious health challenge (9, 20). In the specific case of DFU, its infection may be responsible for amputations, the reduction of the quality life of patient, and, consequently, an increase of the cost of health services. Diabetic foot infections (DFI) frequently require hospitalization of patients and, in the worst case, can also lead to death (26). Statically, more than half of diabetic wounds become infected, and 70% of diabetics with DFI might amputate their leg to improve the healing process (2, 20). Although the micro profile of DFIs varies from patient to patient, studies have shown the most frequently microorganisms include *S. aureus*, *Pseudomonas aeruginosa, Escherichia coli*, *Enterococcus spp., Streptococcus spp.*, *Proteus spp,* anaerobe microorganisms e and others such as fungi (Figure 3) (10, 20, 26). *S. aureus*, *S. aureus* methicillin-resistant and *Pseudomonas aeruginosa* are the prevailing microbial strains that occur in patients with infected wounds (27).

In order to treat the chronic wound infected, as well as to accelerate the healing process, the first priority is to stop progression of the infection using antibiotics administrations. Different antibiotics are available for DFI treatment, but the selection of the adequate antimicrobial compound has to be pondered, based on the pathogenic organisms and the antimicrobial susceptibility patterns (20, 26). In the case of an inappropriate choice of the antibiotic, or an extended application, or even a low concentration of the antimicrobial agent, resistant colonies to antibiotics such as ampicillin, gentamicin and ciprofloxacin (first line antibiotics), can be developed (26, 28). Consequently, along with the ineffective immune response of the diabetic body, DFI control has become an increasing health problem, has made it difficult to select appropriate antibiotics for the treatment (20). In recent years, the resistance of bacteria to antibiotics has become more and more evident and it is a cause for concern in the health sector, reason why new rules had to be implemented in an attempt to minimize this problematic. For all these reasons, the control of wound infections can be challenging, and this is why there is an emerging demand from researches around the world to explore new compounds with antimicrobial properties as potent alternatives to conventional antibiotics (20, 24).

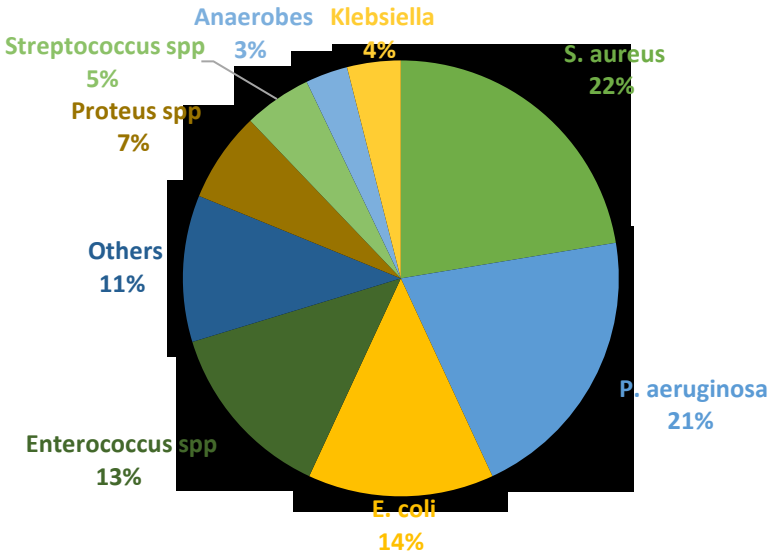

Figure 3- Microorganisms isolated from patients with DFI. Adapted from Saltoglu *et al.* (26)

1.1.3 Current treatment of diabetic wounds and challenges

Currently, there are different available techniques for diabetic wound control including glycemic control, revascularization, hyperbaric oxygen, debridement, administration of growth factors and skin substitutes, pressure off-loading, and application of wound dressings that can be based on antimicrobial compounds, in case infection present (7, 29). However, some of these therapies are expensive, making it impossible to be used in large numbers, since not all hospitals have the resources to implement them.

In the particular case of wound dressings, despite the a regular needed of dressing changes, they are still the most used therapy for wound care due to their low price and flexibility that they provide (30). Nowadays, there are different formulations of wound dressings such as hydrocolloids, films, foams, sponges, membranes, nanofibers and hydrogels (31). Compared all types of wound dressings, hydrogels can be considered one of the best choice for wound application because they are excellent simulators of natural living tissue due to their specific characteristics, such as porosity, flexibility and soft consistency (32, 33).

Hydrogels are made of crosslinked polymers with three-dimensional structure. In addition to a highly moist wound environment, which helps wounds to heal in a faster way promoting the proliferation of fibroblasts, enhancing collagen synthesis and promoting the emigration of epithelia cells (34), hydrogels can also absorb large amounts of water or biological exudates without losing their structure (35, 36). For all that advantages, recent wound management are more focused on moist wound healing environment (37). The goal of any diabetic wound dressing is to control the infection, if it is present, through antimicrobial potential of the material, have proper mechanical properties, be biocompatible and non-toxic, allow gaseous exchange, remove excess exudates, be easily removed without causing trauma, create a proper moisture balance in order to accelerate the healing (20, 22). However, there is not a perfect wound dressing that embrace all necessary requirements (31). In this sense, more studies should be done in order to develop the intelligent wound dressing needed for diabetic wounds (31). The low bacterial properties of hydrogels are considered a significative disadvantage that should be improved in order to increase the biomedical applications (38, 39) As an example, Regranex gel is a commercial available hydrogel for chronic wound that was approved by Food and Drug Administration (FDA), although it has not yet been introduced in Portugal (40, 41). The wound dressing is based on becaplermin, a recombinant platelet-derived growth factor for the treatment of lower extremity diabetic ulcers. However, its non-antimicrobial properties and it is non-sterile are viewed as some disadvantages of the pharmaceutical product. In attempt to overcome the limitation of hydrogels for chronic wounds, antimicrobial compounds, such as antibiotics, are already incorporated in hydrogels in order to improve their antimicrobial properties for wound infections control (42, 43). Similarly, Woulgan®, a hydrogel composed by beta-glucan, is other commercially available wound dressing for chronic wounds (44). The pharmaceutical product is able to promote angiogenesis, cell proliferation and wound contraction, but there are not antimicrobial properties. Bearing in mind that purpose cefazolin (45) and vancomycin (46) are examples of antibiotics that were studied to be incorporated in hydrogels to improve their antimicrobial potential for wound care.

1.1.4 Natural based agents for infection control and healing of chronic wounds

In order to overcome the resistance of microorganism to certain antibiotics previous mentioned, it is urge to discover and study new systems, such as natural compounds with antimicrobial potential, to incorporated in hydrogels, preventing bacterial growth without affecting wound healing process as well as improving the wound response to drugs (20, 26). Different natural compounds were already

studied as potential antimicrobial agents to be incorporated in hydrogels for wound care (47, 48). Currently, some inorganic antibacterial materials, such as silver and zinc oxide nanoparticles, are being studied and used to enhance hydrogel properties (48, 49). Stojkovska *et al*. prepared a silver/alginate nanocomposite hydrogel and *in vitro* functional evaluation was performed, which results shown antimicrobial activity against *S. aureus* and *E. coli* (50). Relatively to zinc oxide, zinc oxide nanoparticles-loaded-sodium alginate-gumacacia hydrogel was studied by *in vitro* wound healing against sheep fibroblast cell culture and antimicrobial tests, showing promising results (51). On a smaller scale, copper is also being studied as an inorganic material with antimicrobial activity to be incorporated in hydrogel for wound care (48, 49). Antimicrobial activity of copper based hydrogel was already tested and proven against *E. coli* and *S. aureus* (52). Chitosan, a natural polymer, was also studied when incorporated in hydrogel and revealed antimicrobial activity against *S. aureus* and *P. aeruginosa* as well as good wound healing properties via *in vitro* and *in vivo* tests (49, 53) However, in terms of wound dressings, CS is normally used to enhance the efficacy of inorganic materials-based hydrogels and reduced the toxicity conferred by them (54). Incorporation of zinc oxide nanoparticles into CS hydrogel for wound care were studied (55). *In vivo* evaluations in Sprague-Dawley rats revealed that the hydrogel enhanced the wound healing and helped for faster re-epithelialization and collagen deposition. Besides, it also shown antimicrobial activity against *P. aeruginosa, Streptococcus intermedius, Staphylococcus hyicus*. In recent years, honey have also shown antimicrobial to improve the healing process of chronic wounds (48). A study evaluated the antimicrobial properties and wound healing activity by *in vitro* and *in vivo* assays of honey-based hydrogel with CS (56). Hydrogel were able to inhibit *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia and Streptococcus pyogenes* and shown great wound healing properties in burn-induced wounds in mice. In addition to all these materials, plant compounds have also spurred interest as a source of antimicrobial agents that can be used on the treatment of chronic wounds (57). Henna *(Lawsonia inemis)* (58), *Aloe vera* (59) and essential oil of *Hypericum perforatum* (60), are examples of plant compounds that are being studied as potential natural antimicrobial agents to functionalized wound dressings for wound healing.

However, besides wound infection, the oxidative stress is also considered one of the most common complications for the delayed wound healing in chronic wounds (25, 61, 62). Skin is continuously exposed to oxidative stress from external factors, producing free radicals and reactive oxygen species (ROS). Due to an unpaired electron in the outermost shell of the nucleus, these species are reactive with high affinity either to donate or obtain electrons from another species to gain stability (25, 63). Skin has its own defense mechanisms to combat that oxidative stress such as enzymes, vitamins and chelating agents, which keeps ROS at very low levels (64). Therefore, under physiological conditions, ROS do not translate into significant damaging effects in the human body. Nonetheless, the persistence of neutrophils during hemostasis and inflammatory phase in wound healing of chronic wounds is responsible for an overproduction of free radicals, which induces oxidative stress and damage of biomolecules (63). Despite scientific advances in natural antimicrobial compounds respond to the need of finding effective alternatives to antibiotics as well as enhancing antimicrobial potential of wound dressings, the incorporation of antioxidant compounds, such as plant compounds, is also absolutely necessary in order to combat the oxidative stress of chronic wounds (65). Plants produce secondary

metabolites such as polyphenols, which have beneficial potentials for the human body and, consequently, have been of great interest for medical and pharmaceutical applications (66). In general, polyphenols are known to have a great antioxidant activity, providing protection against ROS through the neutralization of free radicals by donating an electron or a hydrogen atom (64, 67). For that reason, polyphenols may play a key role in wound care, in particular in wound healing and skin regeneration (57, 64). Some polyphenols have also antibacterial properties. Although the mechanisms of these properties are not yet fully deciphered, it is believed that their activity may be associated to the disintegration of the bacterial cell wall through hydrophobic components of phenolic compounds, the changes in intracellular functions by hydrogen binding of these bioactive compounds to enzymes or by the modification of the cell wall rigidly with integrity losses due to different interactions with the cell membrane (64). Therefore, the enhancement of the lipophilic character of polyphenols increases their antimicrobial activity. Besides, antimicrobial potential of polyphenols was already reported as being special significant against strains resistant to antibiotics, such as methicillin resistant *S. aureus* (27). Tannins are an example of phenolic compounds with antimicrobial potential by inducing damages to the cell membrane and inactivate the metabolism (64, 68, 69).

Thus, it is believed that plant compounds such tannins could be a great antimicrobial as well as antioxidant compound to be incorporated in wound dressings for chronic wounds (65, 70). Currently, polyphenols are already used in different sectors such as pharmaceutical, cosmetic and food fields (66). There are more than 8000 different polyphenols described in literature which are divided into two main subgroups: flavonoids (e.g. flavanols and anthocyanidins), and non-flavonoid compounds (e.g. phenolic acids, tannins, and lignins) (64, 71). There are already some studies that prove the use of polyphenols with antioxidant or/and antimicrobial properties to promote wound healing (Table 1).

Table 1 – Polyphenols with antioxidant or/and antimicrobial properties to promote wound healing

Polyphenol	Materials	Nano/microencapsulation of the polyphenol	Wound dressing	Properties	Study	Tested bacteria	Type of wound	Ref.
Curcumin	Silane	Silane composite nanoparticles	Hydrogel	Antimicrobial	*In vitro* *In vivo*	*Methicillin-resistant S. aureus* *P. aeruginosa*	Burn wounds	(72)
	Gelatin	Gelatin microspheres	Hydrogel	Antioxidant	*In vitro* *In vivo*	-	Diabetic wounds	(73)
	CS, oxidized alginate	-	*In situ* injectable hydrogel	Antioxidant	*In vitro* *In vivo*	-	Not specific	(74)
Thymol	CS and polyethylene glycol fumarate	-	Film	Antimicrobial	*In vitro*	*S. aureus* *E. coli*	Chronic wounds	(75)
	Cellulose	-	Hydrogel	Antimicrobial	*In vitro* *In vivo*	*K. pneumonia, S. aureus, E. coli, P. aeruginosa*	Third degree burn wounds	(76)
	Gelatin, glycerol (plasticizer), glutaraldehyde (cross-linker)	-	Film	Antimicrobial Antioxidant	*In vitro*	*S.aureus, B. subtilis, E. coli P. aeruginosa*	Burn wounds	(77)
	Collagen	-	Film	Antioxidant Antimicrobial	*In vitro*	*S. aureus B. subtilis, E.coli P.aeruginosa*	Not specific	(78)

Kaempferol	-	-	Ointment	Antioxidant	*In vitro* *In vivo*	-	Diabetic wounds	(79)
Carcavol	Clay		Film	Antioxidant Antimicrobial	*In vitro*	*E. coli* *S. aureus*	Chronic wounds	(80)
Chlorogenic acid	-	-	-	Antioxidant	*In vivo*	-	Diabetic wounds	(81)
Resveratrol	Hyaluronic acid and dipalmitoyl phosphatidylcholine	Hyaluronic acid + dipalmitoyl phosphatidylcholine microparticles	-	Antioxidant	*In vitro* *In vivo*	-	Diabetic wounds	(82)
Ferulic acid	-	-	Ointment	Antioxidant	*In vitro* *In vivo*	-	Diabetic wounds	(83)
Tannic acid	Agarose cross-linked with zinc ions	-	Hydrogel	Antimicrobial	*In vitro* *In vivo*	*E. coli*	Burn wounds	(84)
	CS + pullulan	-	Membrane	Antimicrobial	*In vitro*	*E. coli*	Not specific	(85)
	Trimethylolpropane triglycidyl ether (a cross-linker)	-	Hydrogel	Antimicrobial Antioxidant	*In vitro*	*E. coli* *P. aeruginosa* *S.aureus,* *B. subtilis*	Not specific	(86)

1.2.Tannic acid multipotent potential

Tannins are phenolic compounds deriving from a variety of plants as metabolic products. These materials are present in food as well as in beverages, for example in green or black tea and red wine as a flavouring agent (64, 87). Tannic acid is an example of a natural tannin that is present in practically all plants. As other hydrolysable tannins, TA can be found in different beverages like beer, wine and coffee, and in food such as chocolate and bananas, offering an astringent feeling in the mouth (88). This polyphenol is commonly used in the process of leather tanning, in photography as a fixer of dyes, as well as in some pharmaceutical preparations, such as in ointments and suppositories for the treatment of hemorrhoids (89). It can also be used to clear beer and wine, since it can interact with proteins, by forming an insoluble complex (88, 89). Tannic acid (chemical formula $C_{76}H_{52}O_{46}$) is a water-soluble compound and it stands out from the other tannins due to its huge number of phenol hydroxyl groups (90). Its structure is composed by five gallic acids linked to one central glucose molecule. Other five gallic acids are attached to the five gallic acid previously mentioned by ester bond (90). The chemical structure of TA is illustrated in Figure 4. In the presence of specific enzymes like tannase or under alkaline conditions, TA can be hydrolyzed to produce glucose and gallic acid (89, 90). Indeed, based on chemical structure of TA, it is perceptible that the polyphenol has electrostatic, hydrogen bonding, and hydrophobic interactions capabilities, which allows TA to interact with certain biomacromolecule such as chitosan and collagen (91).

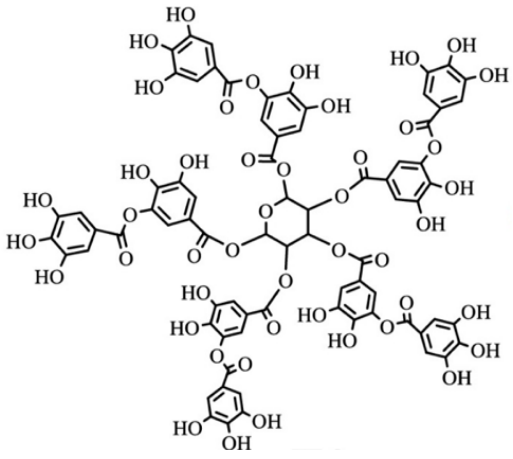

Figure 4 – Chemical structure of TA (90)

In addition to its excellent antioxidant properties, conferred on it for being a polyphenol, TA has also other interesting properties, such as antiviral, anti-enzymatic, hemostatic, anti-inflammatory , anticarcinogenic and antimicrobial activities without cytotoxicity (85-87, 92). In the mid-1920's TA was normally used for the treatment of burns. In the following twenty years, concerns related to hepatotoxic effects of the phenolic compound appeared, in particular when applied in burns in children, resulting on

its disuse. However, a re-evaluation of earlier studies allowed to understand that TA was a safe material for burn application, at certain concentrations (< 5%) (93). Therefore, the interest in the use of TA for wound care reappears (93). Nowadays, TA is considered by FDA as a safe material to be used in food products, but it can be moderately toxic in high quantities by the inhalation and ingestion exposure, inhibiting the absorption of iron in the body and reducing the effectiveness of digestive enzymes (94). In mice, the median lethal dose (LD50) has been reported to be 2.26 g/kg for oral administration but values for dermal or inhalation were not found (94). However, it was already proven that TA can be used as a bioactive compound for the treatment of wounds and skin ulcers (85, 89). As an example, Tanac Liquid is a product composed by benzocaine (active ingredient), and TA is one of the excipients. The pharmaceutical product acts as an oral pain reliever, for oral ulcers and minor irritations of the mouth and gums (95). Another example is 4Jointz®, a natural solution composed by menthol as an active ingredient (1.5%), and, again, TA acts as an excipient. This analgesic cream is applied topically directly to the affected joints, targeting the pain, swelling and stiffness providing soothing relief (96).

To the best of our knowledge, there are no TA-based hydrogels in the market aiming the treatment of chronic wounds. There are some studies about hydrogels that contain TA molecules, where TA is used as cross-linker agent (97, 98) Besides, Nurettin Sahiner *et al*. (86), prepared an antioxidant and antimicrobial hydrogel composed of free TA, using trimethylolpropane triglycidyl ether as a cross-linker, for chronic wounds application. This study was the first and the only one related to a fully polyphenolic hydrogel to be used as wound healing for chronic wounds. The hydrogel shown healing properties in different pH conditions. Additionally, through minimum inhibition concentration values, the wound dressing displayed antimicrobial activity against *P. aeruginosa, S. aureus, Bacillus subtilis* and *Candida albicans*. In terms of healing properties of TA, a very recent study evaluated wound healing properties of free TA by *in vitro* and *in vivo* tests, which results revealed that TA was able to accelerate re-epithelization and to increase hair follicles (99). However, its antimicrobial activity was not studied, and the study is not directed to the specific case of diabetic wounds. In this sense, it is necessary to study its antimicrobial activity against the certain bacteria normally present of diabetic wounds in order to predict if the polyphenol is able or not to be an efficient candidate for chronic wounds treatment. Thus, once TA healing properties are already known and depending on antimicrobial of TA, it is expected a great potential in the incorporation of TA into biomaterials for wound care as a natural antimicrobial agent for diabetic wounds (85).

However, despite all its promising properties, TA has certain limitations which are common to most of polyphenols, including instability, low aqueous solubility, weak bioavailability, light sensitivity and limited membrane permeability (100). Consequently, polyphenols are easily oxidized by light exposure, what makes their use and handling limited, also due to its reactivity as well as a limited absorption and delivery into targeted tissues (101). Besides, the concentrations of phenolic compounds that are effective *in vitro* tests are not often sufficient for i*n vivo* studies (102). Thus, as a mean to overcome these drawbacks, encapsulation of TA, as an efficient drug delivery system for topical administrations, is believed to be the solution to this problem, improving the stability of TA as well as a control for its delivery and release (100-102) As an example of effectiveness of encapsulation technique

for polyphenols, curcumin incorporated in hydrogel for diabetic wounds application was studied (73). Since curcumin has weakness in both bioavailability and *in vivo* stability, as most of polyphenols, gelatin microspheres were used to encapsulate the phenolic compound in order to overcome its limitations. Through microencapsulation, obtained results revealed the capacity to significantly promote skin wound healing of the delivery system with curcumin. Similarly, despite the potential health benefits of resveratrol, the polyphenol is a difficult compound to be incorporated into commercial pharmaceutical products due to its poor water solubility, low bioavailability and chemical instability (103). In this sense, nanoparticle systems using pectin as a wall material, were investigated in order to protect and deliver the phenolic compound (103). Antioxidant results shown that encapsulated resveratrol had higher values of antioxidant activity than free resveratrol.

1.3. Drug delivery systems for topical application

In case of infection, chronic wounds are characterized by having a reduced permeability to bioactive compounds when applied at the wound site (9). That is one of the reasons why delivery systems, aiming the controlled release of carried drugs, like polymeric dressings, are becoming more and more vital in new pharmaceutical products in order to achieve an adequate therapeutic effect in wounds (104). Different drug delivery systems have been developed to release an active agent without side-effects for human body (33). There are different ways of controlled delivery systems, including nanoparticles, microspheres, microcapsules, and liposomes. Independently of their type, all of them have to maintain the stability of the bioactive compound, the releasing of drug control, be biocompatible, as well as the degradability of the material carrier after drug release (87). All advances made in this area allowed to achieve some important objectives related to the proper chronic wound application of compounds, such as antimicrobial agents, including a controlled release of the bioactive compound, reduction of bioactive concentration needed at the wound, and protection of the core material (31, 105, 106). Indeed, skin is composed by four layers which act as a protection barrier against bacteria, virus and fungi. However, skin barrier characteristics also represent a difficulty for the delivery of any bioactive compound in topical application (105). One of the big challenges is to provide an effective concentration on the site of the wound with a prolonged release without causing any damage or toxicity (101, 105). For this reason, microencapsulation, in particular based on biocompatible and natural polymers, is often used to facilitate the delivery of bioactive compounds, such as polyphenols, with a well-controlled release profile for topical application (89, 90, 107).

1.3.1. Microencapsulation of phenolic compounds

Microencapsulation is a technique which particles of one or more bioactive compounds can be stored, coated or surrounded within a shell or a film composed of a polymeric material to produce

microparticles (104). Microencapsulation emerged in the decade of 30s and the first large-scale technical application was related to the development of carbonless copy paper in 1950 (106). The applications of microencapsulation are exponentially increasing due to its high potential, and nowadays there is a panoply of industries where microencapsulation can be applied, such as biomedical, cosmetic, food, veterinary, textile, agriculture, pharmaceutical and chemical industries (71, 100). Microparticles are also receiving a great interest to increase the potential of hydrogel to chronic wounds applications (33, 42). Although there are already several commercial pharmaceutical products based on this technology, natural polymers, like pectin, alginate and chitosan, are being studied to be incorporated in new products as encapsulating materials (100).

An ideal microencapsulation system should provide protection of the encapsulated material against environmental factors including UV rays, moisture and oxygenated environment, increasing its own life and its properties such as reactivity, durability and photosensitivity (71). Additionally, it can also promote a controlled release and a targeted drug delivery; to increase life storage of carried volatile compounds; reduce toxicity; enhance handling properties, application and storage of the encapsulated material; overcome solubility incompatibilities between the core compound and the substance where the capsules are incorporated (100). Regarding morphology, microparticles may have regular (spherical, oval and tubular) or irregular shapes, and its size may range between 1 μm and 1000 μm (71, 100, 108). In terms of morphology/structure, microparticles can be classified as microcapsules or microspheres. Generally, both terms are considered and mentioned as being equal, but, theoretically, they have different morphologic characteristics. While microcapsules are described as a vesicular or reservoir system, where the shell is around the core (or an outside layer), microspheres are characterized as a matrix type, where the core material is distributed homogeneously or heterogeneously into the outside layer (71).

For the development of microparticles, there are certain steps that must be followed. First of all, it is necessary to identify the core, followed by the selection of the coating material and a microencapsulation technique (105). After its production, particles must be evaluated regarding their properties (size and shape), stability, encapsulation efficiency and releasing behaviours guarantee that their formulation process was correctly performed. Scanning electron microscopy (SEM) – morphology characterization – Fourier-transform infrared (FTIR) and differential scanning-calorimetry (DSC) – physicochemical characterization – are common techniques used to particles characterization (71, 105). In terms of mechanisms of drug release in the target site, three phenomena, alone or in combination, may occur: the barrier coat of the microparticle dissolves to release the encapsulated material; the bioactive compound diffuses from de matrix through the outer coat of the particle (100, 104).

Microencapsulation of polyphenols has been continually explored in order to increase their bioavailability in several potentials in food, pharmaceutical and agriculture industries as well as therapeutic potentials in healthcare (109). However, microencapsulated phenolic compounds still have certain limitations that need to be overcome, such as: in case of *in-vivo* treatment difficulties to reach the target organs to perform curative effects, and the volume of reproducible encapsulated phenolic constituents is scarce. The solution might passes by improving and optimizing the encapsulating

formulation of materials as well as the microencapsulation process to increase their affinity towards target organs (100).

1.3.2. Spray drying as a microencapsulation technique

Nowadays, in order to increase the application spectrum of microencapsulation, different microencapsulation methods have been developed and are used in distinct areas. The choice of the most suitable technique must be studied, depending on decisive factors, including the type and the size of particles desired, the application of the microparticles, physicochemical properties of the core material as well as the encapsulating agent, the type of controlled release, the production scale and the cost (104, 108). Spray drying method is one of the most used techniques for the encapsulation of polyphenols (71).

Spray drying is a process based on the transformation of a liquid feed, composed by a certain active agent with a chosen coat material, in dry particles by atomizing the feed in a flow of hot air (110). Although it was first developed in 1860s, the industrial application of spray drying began with the milk and detergent industries in the twenties (111). Nowadays, spray drying has been expanded to different applications, like food and pharmaceutical industries, and it is considered one of the most common procedures for the production of microparticles in large scale (71, 101). The process embraces the following steps: (i) preparation of the liquid feed, where the active compound has to be dissolved, solubilized or emulsified in a solution, suspension or emulsion of the encapsulating agent; (ii) pump the feed solution (previous homogenized) into the drying chamber followed by atomization into a spray; (iii) evaporation of the solvent through the mixing of fine droplets in the drying chamber using hot air; (iv) separation of the particles with diameters between 10 and 500 μm from the drying air (or gas stream) on the cyclone at an outlet temperature of 50-80ºC; and (v) collection of the final dried powder on the collector (71, 110, 112).

Spray drying is a relatively fast, simple and reproducible technology when compared to other methods, and it is also known by the following advantages: stability and quality of the product throughout the entire process, suitability for heat-sensitive and heat-resistant compounds, high encapsulation efficiency, profitability regarding time/cost in a large scale process when compared to freeze-drying; suitability for different types of feedstocks, wide range of dryer designs for specific capacities and applications; and an easy control of microparticles properties (71, 113, 114). Based on all these advantages, spray drying is one of the most used methods to produce microparticles in food and pharmaceutical industry and laboratory applications, as well as the most suitable method for the encapsulation of hydrophilic and hydrophobic phenolic compounds obtained from plant source (71). However, spray drying has the disadvantage of leading to the production of particles with non-uniform shape and size as well as the tendency to aggregate. Another limitation of this technology is the type of the shell material that should be soluble in water (115).

Encapsulation efficiency of spray drying depends on the characteristics of the encapsulating and core material, on the wall/core mass ratio and on the process conditions above mentioned. The total drying time is also a critical parameter: if this value is too high, there is an unnecessary economic loss; if it is low, the process could not be complete. On the other hand, to get the desired size of particles, critical parameters of spray dryer should be controlled, including the inlet and outlet of air temperature (the values of which are directly proportional), atomization pressure, feed concentration and viscosity, and the feeding rate (71, 111, 112). In terms of inlet temperature, the higher it is, the higher the effectiveness of evaporation of the volatile materials at the surface of the capsules and, consequently, the higher the product yield. Normally, inlet temperature values are between 150 and 220 ºC, but the increase of the temperature to higher values can lead to a transformation or loss of carried active compounds, especially volatile compounds as well as a reduction of the final yield. For this reason, the optimal inlet temperature, as well as all parameters above mentioned, is not the same for all procedures, and it is important to find the appropriate values (71, 113).

1.3.3. Natural versus synthetic polymers for microencapsulation

The selection of an appropriate encapsulating material is extremely important to achieve the required final characteristics, such as a desirable encapsulation efficiency and microparticle stability. An ideal choice of the wall material should respect the following criteria: (i) the encapsulating agent should have a low viscosity and should provide a good protection to the core material; (ii) the properties of coated material (porosity and solubility); (iii) the encapsulation efficiency, toxicity, and microscopic properties of the surface of the microparticles; (iv) the compatibility between both materials, where the encapsulating material should not react with the core; (v) the microencapsulation process; (vi) the desired size for the microparticles; (vii) the required application of microparticles and economic factors (71, 104, 115).

Both synthetic and natural polymers can be used as encapsulating agents. Synthetic polymers are produced from non-renewable resources, while natural polymers are available in nature, from a wide array of sources, large amounts (33). Although the production of biomaterials based on synthetic polymers is easier to control, which represents an advantage over the natural polymers, solvents (mainly organic solvents) used in their production are usually unsafe to the human body. On the other hand, natural polymers are usually more biocompatible and are less prone to trigger not an immune reaction, since the degradation products are integrated into the normal metabolism (33, 34, 86). Besides, advantages over synthetic polymers also include their versatility, non-toxicity, be highly structurally organized, low-cost, ease in preparation, compatibility with the environment, being inert to host tissue, biocompatible and biodegradable, and having good mechanical strength (116, 117). For all those reasons there is a clear preference for natural polymers in different sector, such as microencapsulation which makes them an ideal choice for drug delivery systems. Additionally, these polymers have been widely used in biomedical and pharmaceutical applications, like wound biosensors, drug carrier and delivery systems, tissue engineering, wound dressings, gene therapy and so on (30, 87, 118).

In the specific case of polysaccharide polymers, alginate and chitosan are two examples of natural compounds that have advantages over the other natural polymers due to their biodegradable, biocompatible and low toxicity (105). Collagen, hyaluronic acid and chitosan are examples of natural polymers commonly used in microparticulate systems for skin wound dressing and drug delivery (105, 119).

1.3.3.1. Chitosan as a polymeric drug carrier in hydrogels

Chitosan (CS) is a cationic polymer arising commercially from N-deacetylation of chitin, one of the most abundant polysaccharide in nature that is presented in exoskeletons of insects and crustaceans (120). Its chemical structure is characterized by two types of repeated units linked by glyosidic linkage: N-acetyl-d-glucosamine and d-glucosamine. The presence of a large number of amino groups (-NH2) confers chitosan different properties due to its availability for chemical reactions (Figure 5) (121).

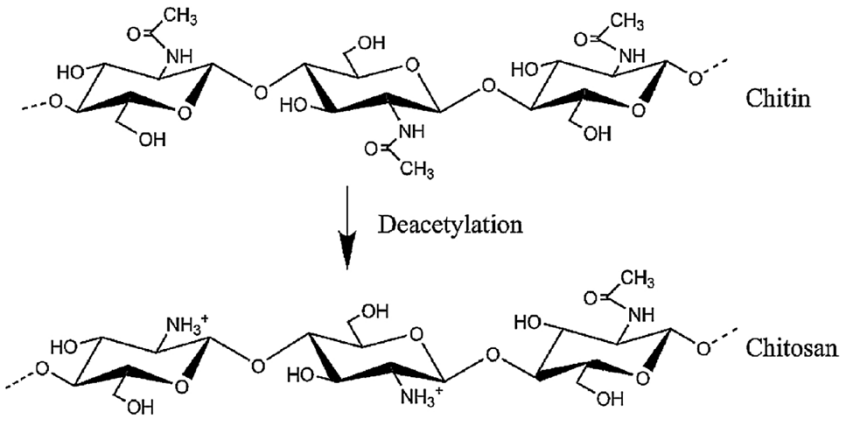

Figure 5 - Chemical structure of chitosan (35)

Chitosan may have different degree values of N-acetylation, the ration of glucosamine to N-acetylglucosamine units may vary from 70% to 98%, and molecular weight, expressed as an average of all the molecules present in the sample (10-100.000 kDa). Both variables can affect the properties of chitosan as a drug carrier such as the speed of its degradation and the microencapsulation process: the lower the deacetylation degree, the higher the biodegradability of CS. On the other hand, the molecular weight is responsible for hydrophilicity, viscosity and biodegradability of CS (115, 122). Chitosan is degraded *in vivo* by enzymes, for instance, glycoside hydrolases such as lysozymes, that hydrolyze linkages between glucosamine-N-acetyl-glucosamine (123-125). Furthermore, it is insoluble in water but soluble in acidic media like acetic acid solution in concentrations from 1% to 3%, due to the protonation of chitosan amino groups. Nowadays, this non-toxic compound considered a safe material by United States FDA, it is one of the most used biopolymers both in pharmaceutical and biomedical applications, including the wound healing area, drug delivery systems and tissue

engineering (120, 121). This bioactive compound can be considered an ideal polymer to be used in hydrogels due to its antimicrobial and antifungal activity, antiulcer, and hemostasis capability, biodegradability, non-toxicity, biocompatibility, low-cost, chemical versatility, and biological adhesion (120, 126). Regarding its potentials in wound care, CS is able to interact with negatively charged of skin due to its positive charge at biological pH. Chitosan-based hydrogels was shown to influence all the four stages of wound healing, by regulating the migration of neutrophils and macrophages, suppressing the infiltration of inflammatory cells, improving the inflammatory function of leukocytes, and accelerating fibroblast proliferation (33, 126, 127). As a polymeric drug carrier, CS is also characterized by its ability to be an efficient drug carrier, having good results in the gradual release of several drugs. Wound healing properties of chitosan microparticles were already tested and proved *in vivo*, which allowed the use of CS as a material wall of microparticles for pharmaceutical applications (33). Although commercial CS-based hydrogels using microparticles were found, commercial chitosan-based wound dressings for chronic wounds are already available, such as Tegasorb® 3M and Chitipack P® Eisai Co (126). Different drugs, such as growth factors, antimicrobial agents, hormones, antifungal, stem cells can be incorporated into CS microparticles in hydrogels in order to improve the efficacy of their properties (117, 126). Chitosan microparticles have offered great promise in topical application and, recently, this polymer has been studied as an encapsulating material for polyphenols (71).

1.4. Innovative biomedical formulations for *in situ* gelling

Recently, to avoid the formation of fluid pockets that enable the proliferation of bacteria, easy powders able to create *in situ* hydrogels have been suggested for wound care (128). Under physiological conditions, the powder is able to transform into a gel-form by different mechanisms, including ionic cross-linkage, temperature modulation and pH change. As advantages, in situ gelling system can cover some limitations of performed gels. This system provides an easily dropped preparations as well as enables a more efficient and focused application in wound site (129). Normally, gelling agents are used, such as genipin, the most used non-toxic cross-linker to produce CS hydrogels (129, 130). In the specific case of CS-based hydrogels, the amino groups and hydroxyl groups present in chemical structure of the polymer can be used as functional groups to react with cross-linking agents, such as genipin for *in situ* gelling (130). However, there are cases where external agents are not needed. A dry-powder spray based on silk fibroin microspheres was explored, for direct application on wounds, such as ulcers, by using dry gelling compounds, like calcium gluconate with alginate that is added to the microspheres powder (131). Alginate is a polysaccharide with wound healing properties that is able to form hydrogels by ionic cross-linking. So, the gel layer will be formed by the wound exudate, which makes this method an easy and economical process for gelation *in situ* (131). A schematic representation of this process is given in Figure 6.

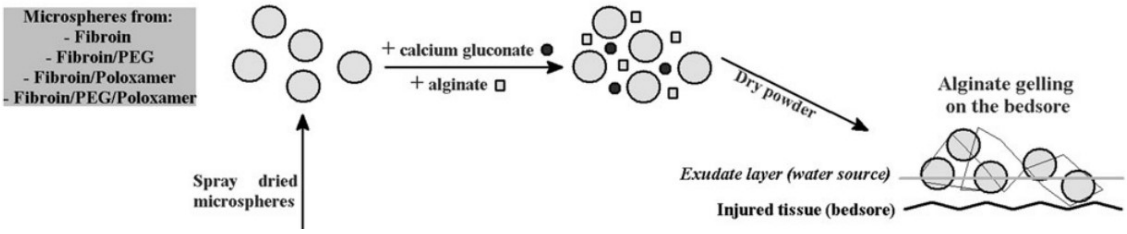

Figure 6 - Schematic representation of auto-gelling process for the fibroin based microparticles dry powder spray (97)

Fibroin is an insoluble protein involved by silk sericin (SS) which confers the structural integrity of silkworm cocoons during its formation (132). Silk is a natural protein fiber obtainable from cocoons produced by the silkworm, such as *Bombicidae*, *Saturnidae*, *Lasiocampidae* or also from spiders. The commercial production of silk is considered one of the oldest activities performed by man, and nowadays, it is produced on a large scale. On its industry, sericin is selectively removed from fibroin during the fabrication process to improve some characteristics of the final product such as the luster, softness, smoothness, whiteness and dyeable fibers (132). Sericin, discarded in wastewater as a by-product, constitutes approximately 50 000 tons out of the 1 million tons of fresh cocoons produced worldwide each year (133). Therefore, the recycling of this protein would have an economic and social positive impact. For this reason, possible applications of sericin have been studied in order to combat that waste (132, 134). Recently, SS has exhibited important biological activities due to its properties such as anti-inflammatory and antioxidant activity, and hydrating properties, making it potentially useful in pharmacological, cosmetic and biotechnological applications, falling within the concept of circular economy (135-137).

Sericin is a globular protein produced by *Bombyx mori*, which represents 20-30% of the total weight of the silk cocoon (132, 138). It is composed by 18 different types of amino acids including serine, glycine and aspartic acid, the most three abundant amino acids present in sericin (137). As a natural protein, SS is characterized by being non-toxicity, biodegradability and biocompatibility (139). Besides, SS has been known to be a protein with great potential for wound care, including its gelling ability, skin adhesion and water-holding capacity (132, 140). Therefore, it is believed that SS is a good candidate for wound dressing material, in particular hydrogels. Lamboni L, *et al* studied a potential hydrogel based on bacterial cellulose functionalized with SS, which shown healing properties enhancing the viability of fibroblast cells (139). Furthermore, a SS-and CS-capped silver nanoparticles-loaded hydrogel was studied to evaluate the antimicrobial and wound properties of the wound dressing. Antimicrobial and wound healing activity of the hydrogel was notable, demonstrating higher bactericidal activity and wound closure when compared to silver-based antiseptics (141). Besides, genipin as well as glutaraldehyde were also reported as cross-linker agents used for generating scaffolds involving SS, for instance, in injectable hydrogels (142, 143). Were studied For this reason, SS can be considered an interest protein to be used in wound dressings, in particular in hydrogels, for chronic wounds treatment (133). In this sense, a group of biomedical researchers

created a patent related to SS-based hydrogel in order to improve wound healing for chronic wound, in particular diabetic wound (144)

Evident advances have been made in hydrogel technology for wound dressing, in order to improve the ability to moisture, coat, protect and fill the wound in depth and shape. Nonetheless and after a thorough literature review, besides hydrogels benefits to the wound bed, they commercially fail in other expectable proprieties that may lead to faster healing rates, like bifunctional activities (i.e. antioxidant, anti-inflammatory, antimicrobial) and drug delivery systems. Moreover, and bering in mind the bacteria resistance to antibiotics as well as the needed of decreasing oxidative stress in complex wounds, the concept of hydrogel as it is now known has to be renewed. Hydrogels with potent antimicrobial and antioxidant properties based on natural compounds, biodegradable and green may be a revolutionary solution in the world of dressing. However, there are still poorly research targeting these bio-based compounds with natural multipotent bioactivities for wound care.

Herein, TA has been explored for its antioxidant and antimicrobial potential in wound dressings, in its free form and encapsulated in CS particles. Microencapsulation is a technique already used to overcome the well-known limitations of polyphenols as well as to improve the release of a bioactive compound. Thus, spray drying was the chosen process for microencapsulation of TA, due to due to its speed and associated efficiency in particle production. In other point of view CS itself, can be a great drug carrier candidate, due to its potential bioactivity in wound care that is already described. Furthermore, in an attempt to reuse by-products of textile industry and, at the same time, to improve wound healing properties of the wound dressing, SS is an interest protein to be used for diabetic wounds treatment to improve tissue hydration and regeneration.

CHAPTER 2

WORK OUTLINE

“Life doesn’t require that we be the best, only that we try our best”

H. Jackson Brown Jr.

CHAPTER 2: WORK OUTLINE

2.1. Aims of the thesis

The aim of this study was to develop and characterize a new powdered wound dressing with antimicrobial and antioxidant properties to apply in chronic wounds, composed by CS, TA, and SS. Tannic acid was encapsulated in CS microparticles by spray drying, and its antimicrobial and antioxidant capacity as well as the profile release of TA were evaluated. Sericin was used to confer regenerative properties and assist wound healing. In this sense, the project focuses on by-products valorisation, in the development of biomedical solutions, in order to increase the economic potential of this technology.

In the future, and after the perform of more studies, it is expected that the present thesis enables a publication of a minireview as well as a research article in a scientific international journal.

To achieve that objective, different steps were established, namely:

- The design a new TA spray dryer encapsulation method, using CS as the polymer matrix;
- The evaluation of the physicochemical properties of the developed CMTA;
- The study of the association efficiency and kinetic profile to assess *in situ* bioavailability;
- The evaluation of the antioxidant and antimicrobial activity of the particles

2.2. Thesis organization

The present thesis is organized in 5 chapters. **Chapter 1** is composed by a theoretical context of the problem under study as well as the reasons why diabetic wounds are a worthwhile problem that must be studied in order to find new strategies to improve their healing process. The importance of natural antioxidant and antimicrobial compounds in wound care is discussed, as well as the multipotent potential of TA and its application in commercial products for wound care. Theoretical concepts of techniques and methodologies such as microencapsulation by spray drying and encapsulating materials used in the present work are explained as well as its applicability to wound delivery systems. Hydrating and healing properties of sericin and its economic impact are presented. At the end of the chapter, a small review of the most innovative biomedical formulations with topical applications for wound care is provided. In **Chapter 2** the aims of the project and the organization of the thesis is detailed as a guideline of the work. **Chapter 3** describes the materials and methodology used, including the preparation and characterization of CMTA, analysis of the controlled release study, and *in vitro* biological properties evaluation. **Chapter 4** presents the results obtained and their interpretation. The microparticles are characterized by product yield, size, shape, structure, association efficiency, and the controlled release under physiological conditions is studied. The

antimicrobial activity of TA is assessed against the five different types of bacteria and Minimum Bactericidal Concentration (MBC) values are quantified, the antioxidant activity of the samples is also analyzed. Finally, the overall conclusions of the developed work as well as the emerged limitations and suggestions for future research are presented in **Chapter 5**.

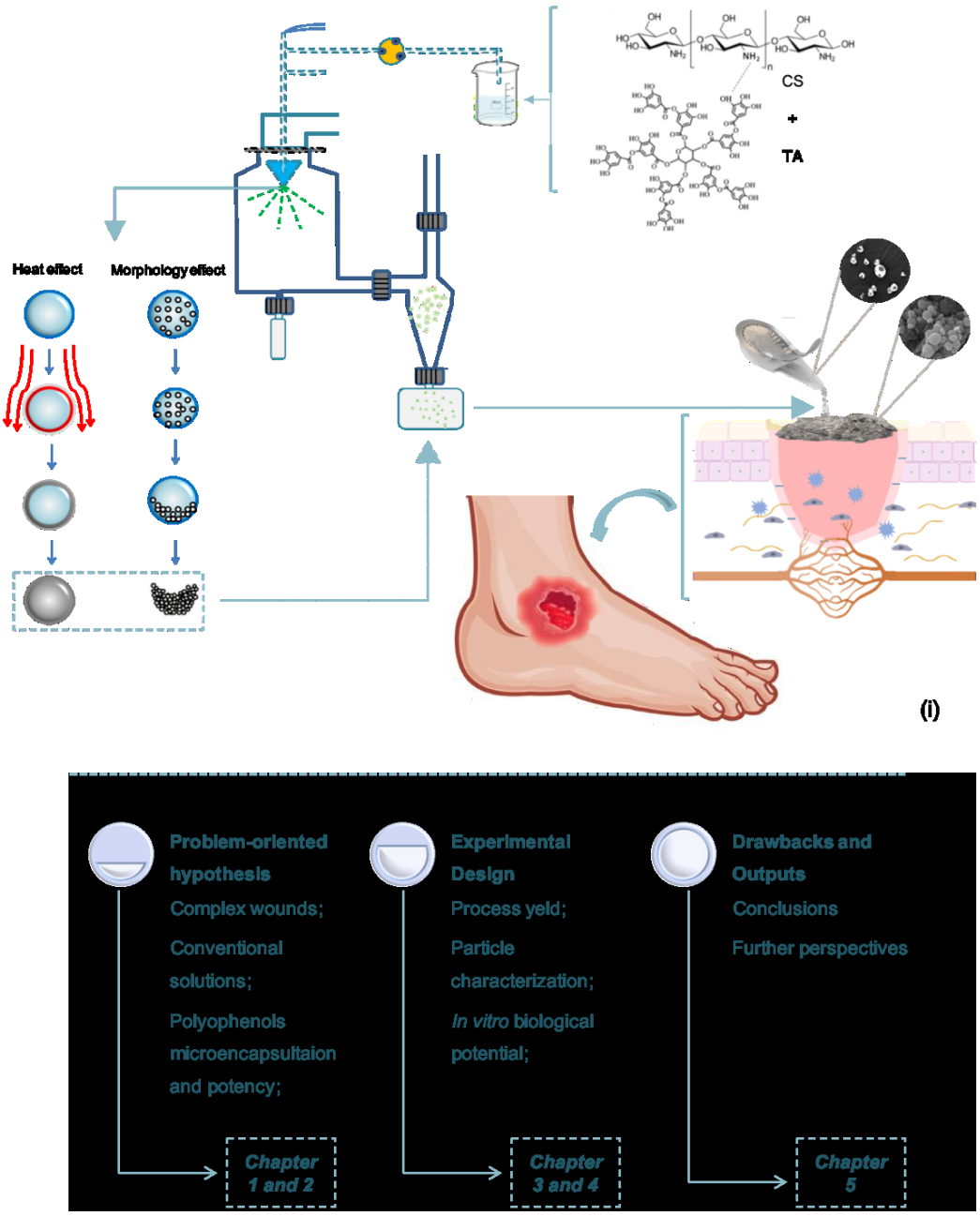

Figure 7 - Scheme of particles production and wound application (i), and general outline of thesis (ii)

CHAPTER 3

MATERIALS AND METHODOLOGY

“If all difficulties were known at the outset of a long journey, most of us would never start out at all”

Dan Rather.

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1. Standards

All standards and reagents, including TA powder, CS of low molecular weight with a viscosity lower than 100 mPa.s and a deacetylation degree of 85%, acetic acid, 2,2-Azinobis (3-Ethyl-benzothiazoline-6-Sulforic acid) and ethanol (96%) were obtained by Sigma-Aldrich (St. Louis, MO, USA). For HPLC analysis, methanol (100%) was obtained from VWR International (Radnor, PA, USA). Commercial sericin was obtained from Swapnroop Drugs and Pharmaceuticals, India. Ultrapure water was obtained in the laboratory using a Milipore Mili-Q water purification equipment (Massachusetts, USA).

3.2. Microorganisms used for evaluation of antimicrobial activities

Stock cultures including: *Staphylococcus aureus* DSM 11729, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* were used for antibacterial activities evaluation of TA free in solution and CMTA. Test organisms were first activated from glycerol by transfer in nutrient broth at 37 °C for 24 h, then streaking on Mueller-Hinton agar (MHA) (Sigma-Aldrich, USA). A single pure colony was streaked on MHA then incubated at 37 °C for 24 h, followed by the experiment.

3.3. Bacterium inoculum preparations for the antibacterial experiments

Loopful of culture from each previously inoculated MHA was transferred into Muller Hinton Broth (MHB) (Biokar, France). The broth was then incubated at 37 °C overnight. Then the concentrations of these suspensions were adjusted with the turbidity of 0.5 McFarland (equal to 1.5×10^8 colony-forming units (CFU)/ml). Turbidity of the bacterial suspension were prepared in sterile saline and measured at 600 nm using a Mini 1240 UV-Vis Spectrophotometer (Shimadzu, Japan).

3.4. Preparation of tannic acid-loaded chitosan microparticles

Chitosan solution was prepared with the concentration of 1% (w/v) in an aqueous solution of 1% (v/v) acetic acid. Tannic acid solution of 6% (w/v) was mixture with CS solution in a proportion of 1:5 (v/v) Both solutions were prepared with deionized water at room temperature, and homogenized protected from light for 1 h before the spray drying procedure.

Microencapsulation was performed using a BÜCHI Mini Spray Dryer B-191. The mixture was fed into the spray dryer under the following conditions: inlet temperature, flow rate, as well as air pressure were respectively set at 115 °C, 3.90 mL/min (17%) and 6 bar. The solution was dispersed

into fine droplets through a 0.7 mm nozzle. The outlet temperature was kept at 60 °C to preserve the compounds stability. The dried powder was collected and stored in falcon tubes, sealed with parafilm, wrapped in aluminum foil, and stored on a desiccator. A schematic illustration is presented in Figure 8.

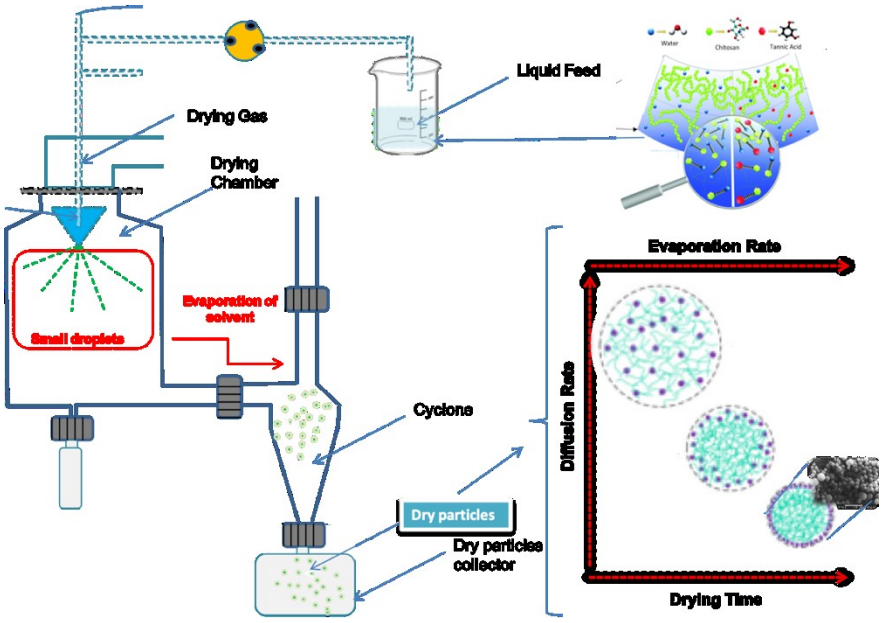

Figure 8 - Spray drying process for CMTA. Adapted from Santos *et al*. (114)

3.5. Physicochemical characterization of chitosan microparticles loaded tannic acid

3.5.1. Product yield

Product yield (%) was calculated for microencapsulation experiment and was expressed as the ratio of the mass of powder collected after drying to the content of the initial infeed solution (Equation 1).

$$\text{Product yield (\%)} = \frac{\text{Mass of powder obtained at the spray dryer}}{\text{Mass of the initial feed solution}} \text{x } 100 \text{ (Eq. 1)}$$

3.5.2. Particle characterization: size distribution and morphology

Particle size distribution was measured by Coulter-LS 230 Particle Size Analyzer (Miami, USA). Before the analysis, suspensions were prepared by adding 0.1% (w/v) of powdered microparticles, following by vortexing. The particles were characterized considering a number distribution. Three replicates were performed. Size distribution was expressed in terms of the mean diameter.

Particle morphology was evaluated by Scanning Electron Microscopy, SEM JSM-5600 LV (Jeol, USA). For samples preparation, powder and hydrated microparticles were mounted onto metal plates and covered with a thin layer of gold under vacuum. In the case of hydrated microparticles, the sample was left at room temperature for a few minutes in order to dry before the analysis. When the samples were evaluated, SEM was operated using 1200 and 4500 x magnification with an electron beam of 30 kV.

3.5.3. Fourier-transform infrared analysis

Fourier-Transformed Infrared Analysis (FTIR) was used to evaluate the structure of TA, CS and CMTA. The structure is generally interpreted through absorption bands based on the specific vibration of the chemical bonds of each substance. Infrared spectroscopy analysis was performed in Spectrum 100 FTIR spectrometer equipped with a horizontal attenuated total reflectance sampling accessory (PIKE Technologies, USA), the Horizon MBTM FTIR software and a diamond/ZnSe crystal. All spectra were acquired using 16 scans and a 4 cm^{-1} resolution in the region of 4000-600 cm^{-1}. Besides that, baseline, point adjustment and spectra normalization were performed. All used samples were run in triplicate, and the data presented were the average of the three measurements.

3.5.4. Differential Scanning Calorimetry

The thermal analysis of TA, CS and CMTA were performed using a differential scanning calorimetry – DSC (DSC-60, Shimadzu, Columbia, USA). 5.0 mg of each sample was crimped in a standard aluminium pan and heated from 25 to 230 ºC at a heating constant rate of 10 ºC/min under constant purging of nitrogen at 20 mL/min. All samples were run in triplicate and data presented were the average of the three measurements.

3.5.5. Association efficiency

Association efficiency (AE) was evaluated considering the amount of TA associated with the microparticles. The AE was measured by the difference between the total TA used to prepare the particles, and the amount of residual TA in the solution immediately after dispersion of the particles in water. AE of TA was obtained according to the following expression (Equation 2):

$$\text{AE}\ (\%) = \frac{\text{Total amount of TA} - \text{Free amount of TA in supernatant}}{\text{Total amount of TA}} \times 100 \quad \text{(Eq. 2)}$$

3.5.6. *In vitro* release of tannic acid from chitosan microparticles

The release of TA from CMTA was tracked to predict the diffusion and kinetic behaviour of the microsystems was tested in simulated physiological environment. For this purpose, 0.1% (w/v) of CMTA were suspended in phosphate-buffered saline (PBS), and transferred to clean Eppendorf tubes, followed by placement in water bath at 37 °C under stirring. PBS was used to simulate physiological conditions at pH 7.4, and its ionic strength was 0.075 M which is in the optimal range for physiological environment proof-of-concept testing and characterization.

Aliquots were collected from the bath over time (0 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h) and centrifugated at 14000 rpm for 5 min (BOECO, Hamburg, Germany). At the end, supernatants were analysed by High Performance Liquid Chromatography (HPLC) to calculate the amount of TA released from the microparticles over the specified time. The quantification was performed by HPLC using the following described method.

3.5.7. High performance liquid chromatography analysis and tannic acid quantification

Chromatographic analysis was performed using the Waters Alliance e2695 Separate Module HPLC. The results were acquired and processed with Empower® 3 Software 2010 for data acquisition (Mildford MA, USA), on an Ace® Equivalence 5 C18 column (250 x 4.6 mm i.d.). The conditions of HPLC analysis were applied according to a method already tested and validated for chromatograms determination of standard phenolic compounds, namely TA, the retention time of which was 4.974 min (145). The mobile phase was composed by two solvents: Solvent A (Acetic acid in water (1:25 (v/v)) and Solvent B (methanol), at a flow rate of 0.8 mL/min. The injection volume was 20 µL and detection wavelength was 280 nm. The gradient program was begun with 100% of Solvent A and was maintained at that concentration for the first 4 min. For the next 6 min, B decreased to 50% and increased to 80% for the next 10 min. At the last two minutes, B reduced to 50% again. Stock standard solutions of TA (10mg/mL) were prepared and used to construct the calibration curve (R^2=0.9983), composed by six standard concentrations of the phenolic compound: 0.02, 0.05, 0.1, 0.2, 0.3, 0.5 mg/mL. The calibration curve is presented in Appendix B.

3.6. *In vitro* biological potential of tannic acid and chitosan microparticles loaded tannic acid

3.6.1. Antioxidant activity assessment

ABTS radical scavenging assay was used to estimate the antioxidant capacity of the encapsulated TA. This method is based on the ability of the antioxidant compounds in solution to capture the ABTS●+ cation, obtained by the reaction between ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) and potassium persulfate. The working solution was prepared by mixing 10 mL of the stock solution of 7.4 mM ABTS aqueous solution and 10 mL of 2.6 mM

potassium persulfate aqueous solution. The mixture was allowed to react for 16 h at room temperature in the dark. The antioxidant potential was measured according to the percentage inhibition, which has to be between 20% and 80% after 6 min of the reaction between 1 mL of diluted ABTS and the sample. The calibration curve was constructed using ascorbic acid in a concentration ranging between 0.010 and 0.100 mg/mL Three replicates were performed for each sample: 1% (w/v) of TA, CS, CMTA, SS, CMTA-SS. The final result was expressed as equivalent concentration of ascorbic acid (in g/L), using the calibration curve.

3.6.2. Antibacterial potential

Antibacterial activities of TA and CMTA were evaluated using well diffusion method on MHA. The inhibition zones were reported in millimeter (mm). *Staphylococcus aureus* DSM 11729, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* were used as references for the antibacterial assay. Mueller-Hinton agar plates were inoculated with bacterial strain under aseptic conditions and wells (diameter = 6 mm) were filled with 50 µl of the test samples and incubated at 37 °C for 24 h. After the incubation period, the diameter of the growth inhibition zones was measured and compared. A schematic representation of the method is given in Figure 9.

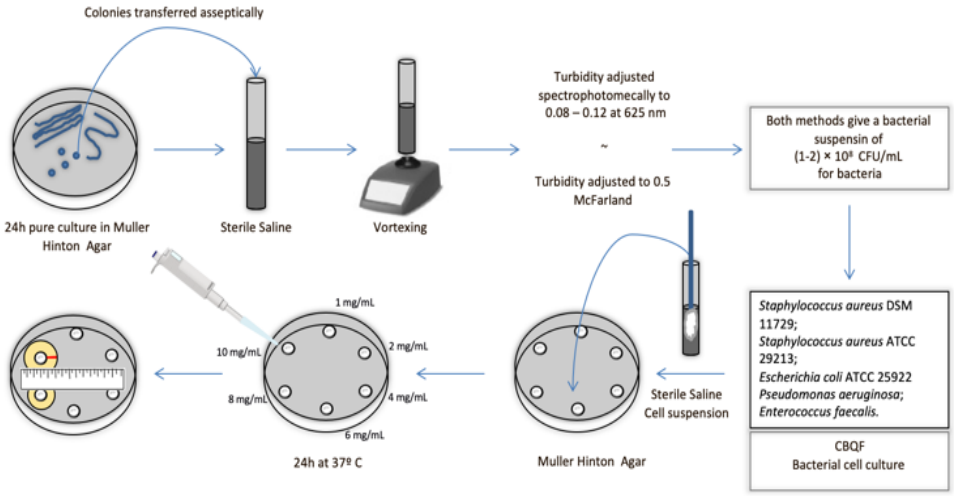

Figure 9 - 0.5 McFarland microbial inoculum preparation by the direct colony suspension, followed agar well diffusion technique for TA concentrations between (1-10 mg/mL)

The lowest concentration of the TA and CMTA that kills > 99.9% of the initial bacterial population showing no colony on the MHA after 24 h of incubation at 37°C was recorded as the MBC. TA solution was tested in different concentrations 1, 2, 4, 6, 8 and 10 mg/mL in ringer and added to Eppendorf tubes with 2% of bacterial suspension in MHB. Three replicates were performed for each strain and inoculated at 37 ºC for 24 h. Then, a drop of 20 µL of each solution, previously prepared,

were transfer to agar plates. After incubation for 24 h at 37 ºC, the results were observed, and MBC of TA was determined. A representation of the method is given in Figure 10.

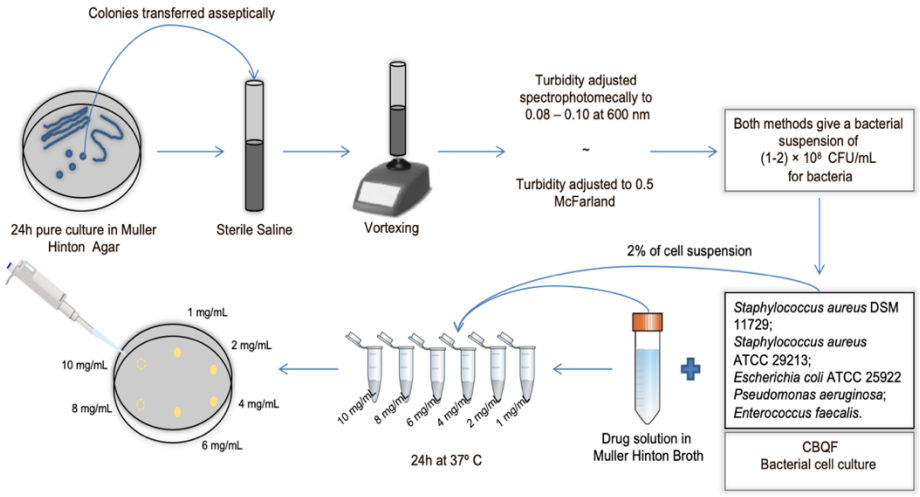

Figure 10 - 0.5 McFarland microbial inoculum preparation by the direct colony suspension, followed MBC technique for TA concentrations between (1-10 mg/mL)

3.7. Statistical analysis

Statistical analysis was performed using IBM®SPSS®Statistics software, version 20.0. In order to evaluate the differences in antioxidant levels of TA, CS, SS, CMTA and CMTA-SS, t-student test was used. Differences were considered to be significant at a level of $p < 0.001$.

CHAPTER 4

RESULTS AND DISCUSSION

“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.”
Marie Curie

CHAPTER 4: RESULTS AND DISCUSSION

Chitosan microparticles loaded TA were characterized regarding their physicochemical properties, including the size, morphology, FTIR and DSC analysis. To simulate wound delivery conditions and to predict the behavior of microparticles over a spaced period of time, until 24 h, a controlled release study of TA encapsulated was performed. Besides that, the product yield of the microencapsulation technique and the association efficiency of microparticles were also evaluated. All these results and their discussion are presented in subchapter 4.1: physicochemical characterization of CMTA. The following subchapter is also dedicated to *in vitro* biological potential of TA and CMTA. In this session, results of antioxidant activity evaluation of TA encapsulated and free in solution are compared. The antioxidant potential of SS when mixed with CMTA were also quantified in order to predict if there could be a synergy effect between the protein and the CMTA developed. Continuously, a qualitative analysis of antibacterial potency of TA (1, 2, 4, 6, 8, 10 mg/mL) against *S. aureus* DSM, *S. aureus* ATCC, *E. coli*, *E. faecalis* and *P. aeruginosa* were assessed. After that, antibacterial properties of the same compound were analyzed qualitatively, where MBC were determined.

4.1. Physicochemical characterization of chitosan microparticles loaded tannic acid

4.1.1. Product yield

Product yield of spray drying was calculated in order to predict the efficiency of the method in the production of CMTA. The product yield obtained for this process was 32%. Although product yield values of spray drying process may be higher than 50%, recent studies reported, for polyphenols encapsulation into CS microparticles by spray drying, values from 29.63% to 57.3% (146-148). According to these works, product yield obtained is considered a satisfactory value, for the laboratory scale and for the materials that were used.

Besides the natural loss of final product associated with adherence of powdered microparticles to the cyclone walls, the solid losses since small particles are suctioned by vacuum filter, and the inability of the separation devices to collect the smallest particles (114, 148), the yield values might also be affected by the type of encapsulating material. During the microencapsulation process, the adherence of CS to the drying chamber wall was observed, probably caused by its natural viscosity. The viscosity of the initial solution should be the lowest possible to allow homogenous pumping of the solution and the atomization (115). In this sense, the CS chosen was the low molecular weight instead the medium molecular weight. Still its viscosity might not be the ideal for spray drying process. Other way to control the physical properties can be related to the feed temperature: the higher the inlet temperature, the lower the viscosity of the solution and, consequently, the better the conditions to increase the yield value. However, higher values of that temperature may be responsible for degradation of some heat-sensitive compounds, like polyphenols

(i.e. TA). So, the choice of inlet temperature value was chosen according to the temperature that can be used safely without damaging the compound (149). Besides the temperature, the pump rate can also significantly affect the yield of spray drying. According to Plamen D. Katsarov *et al*, the lower the pump rate of CS solution, the higher the yield of the process, since the quicker the solution is sprayed, the more energy is needed to evaporate the solvent from the particles (150). Therefore, more experiments should be performed in order to increase the product yield of spray drying, regarding the encapsulation of TA, through the use of other inlet and outlet temperatures, the flow rate and, consequently, the time of spray drying process.

4.1.2. Particles characterization: size distribution and morphology

Control of the size and morphology of microparticles is considered an indispensable analysis due to their influence in the sustained and controlled release of encapsulated agents and microparticles stability (113). Results of size distribution in number of CMTA microparticles are presented in Figure 11. The results showed that microparticles had a mean diameter of 7.4 µm. This result is concordant to the size of microparticles usually produced by spray drying (1-50 µm) (109, 115). The range of values between 25 µm and 53 µm showed to be discrepant results, probably due to an agglomeration zone of microparticles, as can be observed from the following SEM images (71, 151). Therefore, this range was not considered for the calculated mean of the size distribution. Particles with a relative low size values, within micrometric scale has considerable advantages. In addition, despite the fact that microparticles provide a slower extracellular drug release due to a low surface-to-volume ratio, small sized particles may provide a larger surface area, improve the active compound penetration into wound bed as well as promote intracellular uptake (14). Therefore, it is believed that the size of obtained microparticles by spray drying is appropriate for wound care.

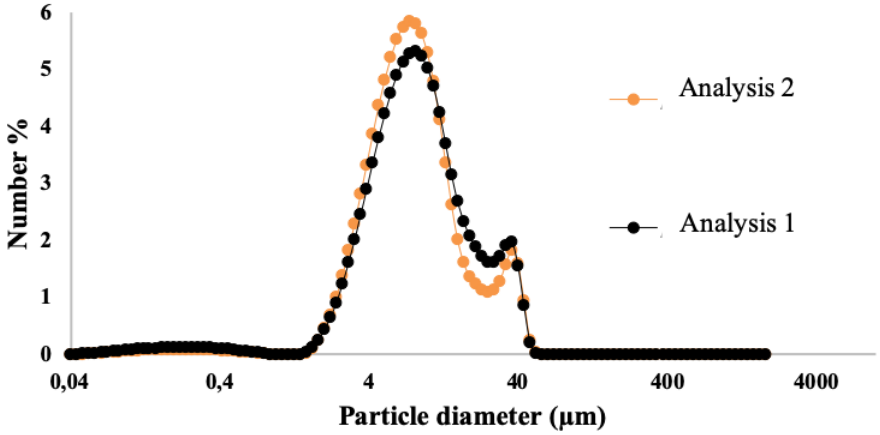

Figure 11 - Size distribution in number of CMTA microparticles

In order to evaluate the CMTA microparticles morphology, SEM images of powdered and hydrated particles are presented in Figure 12.

Powdered chitosan microparticles exhibited spherical shape with some concavities on the outer surface. Concerning hydrated particles, that naturally become swollen after immersion, SEM images showed aggregated microparticles with spherical and regular shape, with a smooth surface. The observed tendency to agglomerate was expectable due to the microencapsulation process used (71). According to some studies, the type of morphology obtained for powdered microparticles, also called “raisin-like”, showed to be typical for CS microparticles as well as CS microparticles loaded polyphenols, produced by spray drying (147, 152-154). This roughness and recesses of particles may be caused by the rapid evaporation of drops of liquid during the drying process in the atomizer, and even by the interaction of amino groups of CS (positively charged groups) within the polymer itself (147, 155). Traditionally, the surface of microparticles is normally smooth, but, although there is little information about the impact of the surface on the release efficacy, it is believed that a rough surface with some concavities might be favorable for tissue healing and cell growth due to the similarity of its structure to the extracellular matrix network as well as to a strong surface adhesion (156). Therefore, regardless how the microparticles would be applied in practice, collapsed or swelled, their morphology in both cases can be considered appropriated for wound application.

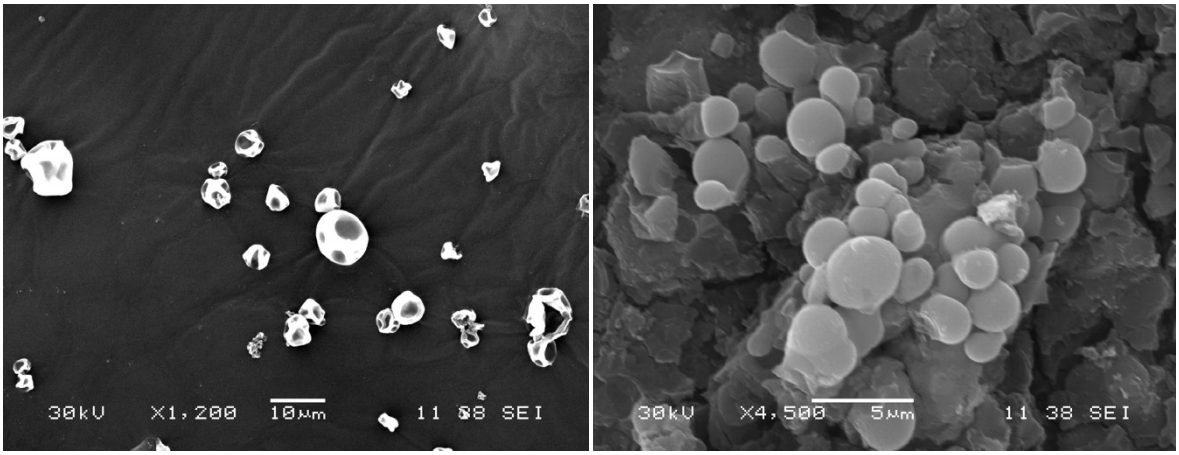

Figure 12 - SEM images of CMTA (A) and hydrated CMTA (B). Magnification of 1200 and 4500 times for A and B, respectively, with beam intensity 30kV

4.1.3. Fourier Transform Infrared spectroscopy

Depending on the structure of a compound, its functional groups produce characteristic absorption bands in the spectrum, which are analyzed by FTIR. With these bands, it is possible to draw conclusions about the possible chemical interactions, namely covalent bonds, between compounds. Therefore, FTIR analysis of CMTA, its substrates and the physical mixture between CS and TA were measured. The results are presented in Figure 13.

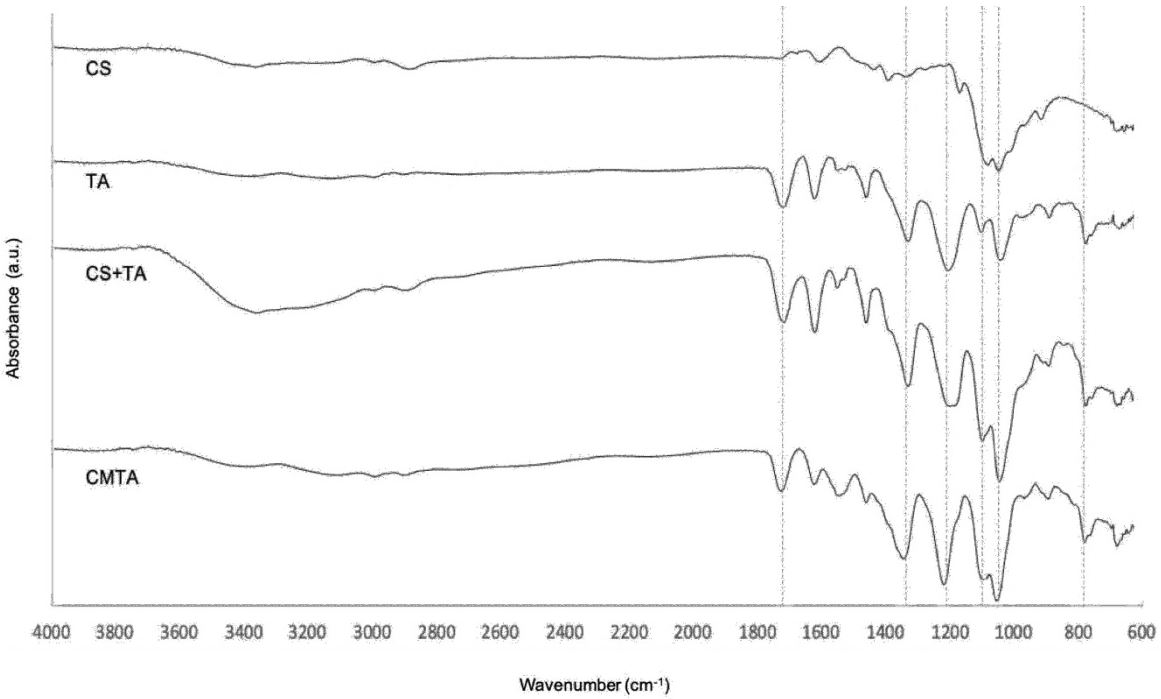

Figure 13 - FTIR spectra of CS, TA, CS+TA and CMTA

Chitosan displays a typical vibrational absorption bands between 1595 and 1308 cm-1 that are attributed to the stretching of specific bonding of amides. However, the peaks presented on CS spectra are not defined, when compared to reported results. The C-O stretching was identified by the presence of peaks at 1057 and 1021 cm-1. At the end, the band located at 896 cm-1 is attributed to the stretching of the glyosidic bond (157, 158).

Tannic acid spectra was consistent with values given by other studies (86, 159). Its FTIR spectrum exhibited characteristic bands of aromatic rings in the wavenumber range of 1445-1698 cm-1. The two bands around 1314 and 1180 cm-1 resulting from O-H and C-O stretches. The vibration of C=C in benzene rings was identified at 757 cm-1.

Spectrum of CMTA microparticles showed that all the above characteristic maintained at the same wavenumber, indicating no interaction between the drug and carrier. These results are concordant with previous study (158). Besides that, no new peaks appeared in CMTA microencapsulation spectrum, as well as in physical mixture of the substrate, indicating that no new covalent bonds were detected from the CMTA production. Therefore, the integrity of TA is expected even after the microencapsulation process.

4.1.4. Differential Scanning Calorimetry

Differential Scanning Calorimetry analyses were performed in order to provide information about physical and chemical changes of CMTA microparticles that involve endothermic and

exothermic variations. The graphs of heat flow (J/g), depending on the temperature (°C) of CMTA microparticles and its substrates are illustrated in Figure 14.

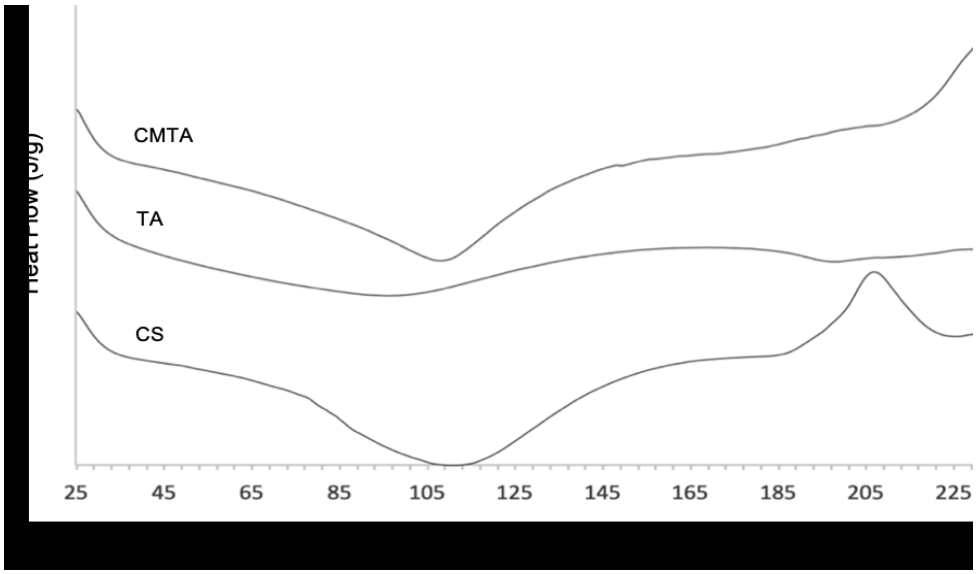

Figure 14 - Heat flow vs temperature of CMTA, TA and CS

The results showed a broad endothermic band between 93.69 and 120.67 °C for CS and an exothermic peak 203.20 and 211.70 °C. As other previously studies reported, the endothermic peak, corresponding to a transition that absorbs energy, endorsing the loss of water related with hydrophilic groups of CS (160). Although CS was in powder form, it might have some associated humidity that gave rise to this peak. In turn, the exothermic one, corresponding to a transition that releases energy, is assigned to the thermal degradation of the polymer or melting transition temperature. That degradation may occur due to glycoside bond cleavage or monomer dehydration (160).

Thermogram of TA exhibited a very broad endothermic band at 96.07 °C, related to the evaporation of hydration water molecules, as reported from literature (161). Besides that, TA did not show any defined peak, which reveal that the phenolic compound is thermally stable from 25 to 230 °C. At tested range of temperature, it was not possible to detect the band assessed to thermal degradation, but it was reported that the degradation of this phenolic compound occurs around 260 °C (158).

Chitosan microparticles loaded with TA displayed a sharper endothermic transition on the same values of CS thermogram, due to water that remains after the spray drying process. Its degradation temperature was not detected at tested temperatures, but it can be observed that possibly an exothermic peak would occur above 230 °C. According to Yingju Jing *et al.* (158), who studied the interaction between TA and CS in order to functionalize CS, the decomposition peak of TA with CS appeared around 280 °C. Therefore, more assays would be needed at a higher temperature range in order to understand when the degradation occurs. However, based on obtained results, the interaction between TA and CS led to an increase in microparticles thermal stability compared to its substrates.

4.1.5. Association efficiency

Tannic acid association efficiency was 98.50% ± 0.02. Recent studies showed values of AE ranging from 52.7 to 92.6% for polyphenols encapsulated in CS microparticles by spray drying (146, 147). Therefore, AE of the microencapsulation process used in the present work is considered an excellent result when compared to reported values and considering the viscosity of the liquid feed caused by CS. Besides the properties of encapsulating material, pH upon microparticles formation could also have some influence on the AE values for encapsulation of TA in CS microparticles. Chitosan in acidic media can interact with negatively charged groups due to the protonation of chitosan amino groups. Therefore, the polyphenol and the polymer may interact with each other through bonding between hydroxyl groups (-OH) or carboxyl groups (-COO) of TA and hydroxyl (-OH) or amino groups (-NH3) of CS (162, 163). A possible interaction between TA and CS is illustrated in Figure 15. It is believed that there is a higher tendency of interaction with -NH3 because, for CS in acidic media, the positive regions concentrate on the protonated amino group (162, 163). Since that reversible interaction through noncovalent bonds is stronger with lower pH values, it is expectable that the acidic media of feed solution, as well as the low molecular weight of CS, are conditions that may positively influence the amount of TA in CS microparticles produced by spray drying (71).

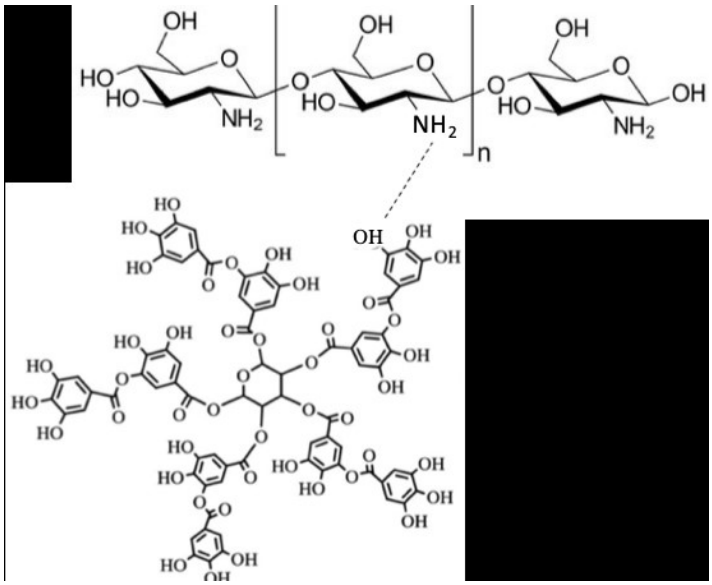

Figure 15 - Possible reversible interaction between -OH and -NH3 of TA and CS, respectively.

4.1.6. *In vitro* release of tannic acid from chitosan microparticles

The controlled release of TA was evaluated in physiological conditions to assure the desirable time and rate in wound bed. Topical delivery conditions were simulated in PBS over a period of time of 24 h. Results showed a controlled release profile. The release of the core material depends on the

type of the encapsulated material, the core-to-coating proportion, as well as the environment where microparticles will be implemented (71).

The peak of TA (6.611 min) was similar to values reported on the study which describes the method used of TA detection (145). The quantification of the phenolic compound was quick and easily performed. A representative chromatogram of TA is shown in Figure 16.

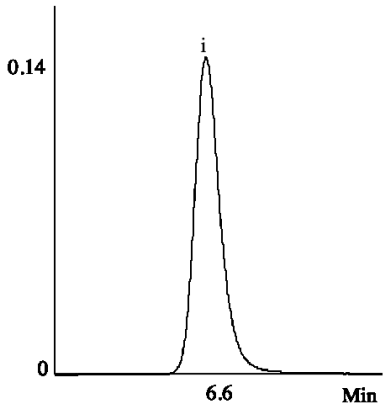

Figure 16 - Representative chromatogram of TA (i), and its retention time: 6.611 min

According to obtained results presented in Table 2, 0.77% ± 0.002 of the total encapsulated TA was released on the first time point (T0 h), and 0.77% ± 0.003 on the following 24 h (T24 h). The amount of released TA was practically the same (≈0.8%) during the first 24 h.

Table 2 - Chromatogram of CMTA after 24 h of release in PBS, analyzed by HPLC

Time points	TA release (%)
$T_{0\,h}$	0.77% ± 0.002
$T_{0.5\,h}$	0.72% ± 0.004
$T_{1\,h}$	0.72% ± 0.004
$T_{2\,h}$	0.72% ± 0.003
$T_{4\,h}$	0.79% ± 0.005
$T_{6\,h}$	0.81% ± 0.105
$T_{8\,h}$	0.82% ± 0.080
$T_{24\,h}$	0.77% ± 0.003

Results seem to indicate that, probably, the TA content entrapped in the core of the particles was not successfully released in 24 h. Although further testing would have to be done to secure this premise, a possible justification for the obtained values may be related to the amount of TA that can remain on the microparticles surface after spray drying process (71). In other words, the amount that was read in HPLC might be the portion of polyphenol in the microparticles surface, which means that the microsystem did not even depredated to release TA. The non-degradation of microparticles might be associated to the conditions of the release medium, in particular alkaline pH values of PBS. According to Neculai Aelenei *et al.*, the release of TA is significantly lower in pH values higher than 7.4 such as PBS, due to the partial insolubility of CS in an alkaline medium (89). However, in acidic medium, more than 90% of the encapsulated TA is released during the first 20 h. The pH values of diabetic wound/chronic wounds are typically alkaline (from 7.2 to 8.9), which hinder the healing process and create a great environment for the growth and multiplication of bacteria (164). In contrast, lower pH values on the chronic wound surface provide an acidic environment, which helps the wound healing by controlling wound infections (165). In this sense due to the alkaline pH of complex wounds, the TA release is expected to be slow over time. In a clinical point of view, this lagging release may allow its bioactivity control for 2 to 3 days, until the wound dressing is replaced by medical or nursing services. This may represent a time-regulated regeneration and infection control. Nonetheless, the chronic wound environments are also characterized by containing degradative enzymes, for instance, lysozymes or proteases (127). These enzymatic phenomena may promote a fast release of TA entrapped within the core of CS particles, due to an erosion of the encapsulating material (CS) (24). Therefore, and regardless the slow kinetic profile of TA in this assay, it is believed that a fast release could happen *in situ,* by the enzymatic and inflammatory cascade typical in the wound bed (123). Once more and to guarantee the veracity of the obtained results, more studies and tests should be done. Studied CS particles Further experiments could be done simulating enzyme degradation in PBS, using Protease XIV from *Streptomyces Griseus* at concentration of 3.2 U/mg and temperature (37 ºC) according to previous works (166).

4.2.*In vitro* biological potential of tannic acid and chitosan microparticles loaded tannic acid

4.2.1. Antioxidant activity evaluation

The antioxidant activity of TA free in solution and encapsulated into CS microparticles was analysed using the ABTS radical scavenging assay. Besides, antioxidant activity of SS and CMTA-SS were also evaluated. The results are present in Table 3. All samples were analysed in 1% (w/v).

Table 3 - Antioxidant activity of TA encapsulated into CS microparticles and free in solution

	ABTS (eq. [Ascorbic acid] g/L)
TA	22.69 ± 0.62
CS	0.03 ± 0.00
SS	0.09 ± 0.00
CMTA	18.79 ± 1.04
CMTA-SS	6.39 ± 0.09

As a polyphenol and, consequently, a powerful antioxidant compound due to its abundant phenolic hydroxyl groups, the best antioxidant activity results (22.69 ± 0.619) were obtained for TA (1%) free in solution. As expected, CS had a much lower antioxidant activity due to its insufficient H-atom donors, as well as SS (0.03 ± 0.00 and 0.09 ± 0.00, respectively) (158). Comparing to TA free in solution, encapsulated TA showed a significant reduction ($p < 0.001$) of antioxidant activity (18.79 ± 1.04), certainly caused by the entrapment of the polyphenol into the microparticles. However, the microencapsulation of TA does not eliminate its antioxidant activity, which is concordant with a previous study related to the microencapsulation of polyphenols using CS as a microcarrier (147). Despite that significant decay, antioxidant activity of CMTA is still higher when compared to TA free in solution, probably due to the amount of TA that remained on the microparticles surface after spray drying process (71). Relatively to the incorporation of SS into CMTA dried microparticles, the antioxidant potential of the powder decreased compared to the powder without SS. That difference of values can be ascribed to the possible tendency of interaction of SS with TA through hydrogen bonding between hydroxyl groups of TA and hydroxyl, amino or carboxyl groups of SS amino acids. The interaction polyphenol-protein was already reported and can be affected by temperature, pH, type and concentration of protein and type of structure of the phenolic compound (167). However, information of specific interaction between SS and TA was not found in the literature. Therefore, in spite of its beneficial properties for wound care, SS may be reduced in future formulations of the powder of microparticles, once antioxidant activity of TA is significantly affected.

4.2.2. Antibacterial potency

Agar diffusion perfusion method was used in order to analyze the antimicrobial activity of TA qualitatively against all five studied bacteria: *S. aureus* DSM*, S. aureus* ATCC*, E. faecalis, P. aeruginosa and E. coli.* All the results are plotted in Table 4, where the standard deviation was calculated from the triplicates performed for each experiment.

Table 4 - Results of inhibition bacterial growth zone (mm) produced by different concentration of TA (1-10 mg/mL) against *S. aureus* DSM*, S. aureus* ATCC*, E. faecalis, P. aeruginosa and E. coli*, by well agar diffusion agar

	1 mg/mL	2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL	10 mg/mL
S. aureus DSM	0.00 ± 0.00	1.50 ± 0.50	2.50 ± 0.50	2.50 ± 0.50	3.50 ± 0.50	4.50 ± 0.50
S. aureus ATCC	0.67 ± 0.94	1.33 ± 0.47	2.67 ± 0.94	3.33 ± 0.47	3.33 ± 0.47	4.33 ± 0.47
E. faecalis	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	4.00 ± 0.00
P. aeruginosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E. coli	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

The obtained values demonstrated that TA was able to inhibit both types of *S. aureus*: *S. aureus* DSM from the concentration of 2 mg/mL, and *S. aureus* ATCC of the minimum tested concentration (1 mg/mL). Furthermore, TA had shown an inhibition zone against *E. faecalis* of the concentration of 4 mg/mL. It is also possible to observe that, in most cases, the higher the concentration of TA, the higher the diameter of the inhibition zone. However, the phenolic compound was not able to inhibit the growth of *P. aeruginosa* and *E. coli*, both Gram-negative bacteria.

There are different mechanisms proposed to justify tannins antimicrobial potential, such as changes in the intracellular functions caused by hydrogen binding of tannins to enzymes, what leads to an extracellular enzyme inhibition and unavailability of substrates for digestion (168). However, it was shown that the primary site of their inhibitory action is the microbial cell membrane on causing morphological changes of the cell wall through interaction with proteins, which lead to a precipitation in them and, consequently, an increase of the membrane permeability and microorganism death (168, 169). The different behaviors of polyphenols between Gram-positive and Gram-negative are still a controversial issue. It is known that the cell wall is mainly composed by peptidoglycan (68). Results from a study from Guofeng Dong *et al*. (68) revealed that TA is able to link to peptidoglycan of the cell wall and it may inhibit the formation of the biofilm. However, a Gram-negative bacterium has an outer membrane layer composed by lipopolysaccharide molecules and phospholipid that is external to the peptidoglycan cell wall. It was proven that *SA* was more susceptible to tannins than *E. Coli*, due to the lipopolysaccharide molecules negatively charged on the outer membrane. Therefore, normally, tannins have been more effective against Gram-positive bacteria than Gram-negative, which is concordant with the obtained results (68, 69, 170).

Since the agar well diffusion test only gives qualitative results, and the minimum inhibitory concentration (MIC) was not successful achieved by turbidimetry due to instantaneous precipitation of TA in the tested liquid media, qualitative MBC values of antimicrobial TA were assessed to complete the antibacterial analysis. The results are presented in Table 5.

Table 5 - MBC results of TA (1-10 mg/mL) against *S. aureus* DSM*, S. aureus* ATCC*, E. faecalis, P. aeruginosa and E. coli*

	1 mg/mL	2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL	10 mg/mL
S. aureus DSM	+	+	+	+	-	-
S. aureus ATCC	+	+	+	+	-	-
E. faecalis	+	+	+	+	+	+
P. aeruginosa	+	+	+	+	+	+
E. coli	+	+	+	+	+	+

* + represents bacteria growth; - represents bacteria inhibition

Minimum bactericidal concentrations of TA against *S. aureus* DSM and *S. aureus* ATCC were both 8 mg/mL, which means that 8 mg/mL is the lowest concentration of TA that kills 99.9% of bacteria. The results of *P. aeruginosa* and *E. coli* were consistent with the results of the previous assay, which means that TA had no effect on the growth of *P. aeruginosa* and *E. coli*. However, although TA showed inhibition effects on growth of *E. faecalis* with agar perfusion method, its MBC value was not found in tested concentrations. This discrepancy of results may be due to the fact that *E. faecalis* biofilm is characterized by having a very clear color, which might have resulted from a misperception when measuring the diameters of inhibition growth zones in the qualitative method. On the other hand, MBC value of TA against *E. faecalis,* and even against *P. aeruginosa* and *E. coli*, can be higher than tested concentration because the inhibitory effect of TA is depending on the concentration of the tannin to which the bacteria are exposed. Therefore, it is considered that TA has no antimicrobial activity against these three bacterial strains at tested concentrations.

Results of antimicrobial activity CMTA microparticles with SS are present in Table 6.

Table 6 - Antimicrobial activity of TA, CS, CMTA, SS and CMTA-SS

	C-	TA	CS	CMTA	SS	CMTA-SS
S. aureus DSM	+	-	-	-	+	-
S. aureus ATCC	+	-	-	-	+	-

* + represents bacteria growth; - represents bacteria inhibition

According to the results of both antimicrobial methods previously presented, *E. faecalis*, *P. aeruginosa* and *E. coli* were excluded from the study due to non-susceptibility to the highest concentration tested of TA. Therefore, the microbiological *assay* was only performed for *S. aureus* DSM and *S. aureus* ATCC, the most prevalence of microorganisms in DFU. Results of TA were

concordant with the results presented in Table 4. The polyphenol and CS (1%) were able to kill both *S. aureus*. Antimicrobial activity of CS against *S. aureus* was already reported (157). Its main underlying mechanism is related to the linkage of positive charged amino groups (NH_3^+) and the negatively charged molecules such as proteins, anionic polysaccharides and nucleic acids in bacterial membrane, leading to altered membrane permeability with the release of cellular contents, causing cell death (126, 171, 172). Relatively to encapsulated TA, CMTA also showed positive values against *S. aureus* DSM and *S. aureus* ATCC, due to a possible synergy between the of CS with the possible released TA caused by hydration of microparticles. The synergy between antimicrobial activity of CS and antimicrobial activity of certain polyphenols, such as caffeic acid (173), ferulic acid (174) and hydroxycinnamic acid (175) were already studied and validated. However, SS did not exhibit antimicrobial potential against no type of bacteria, but the protein did not compromise the activity of CMTA.

CHAPTER 5

CONCLUSION AND FUTURE POSPECTS

“Everything seems impossible until it is done”
Nelson Mandela

CHAPTER 5: CONCLUSION AND FUTURE POSPECTS

5.1. Conclusion

In a trend-driven scientific and technological world, biomedicine has been evolving exponentially in various fields of knowledge to meet rising average life expectancy and the need for increasingly efficient and specific pharmaceutical solutions. However, systemic and widespread infections are still amongst the most common causes of death worldwide. It is unsettling that sometimes infections may start in simple processes as an exposed and poorly healed wound. In case of diabetes, an additional problem is compounded by the metabolic compromise, natural delayed healing and consequent susceptibility to infection. This is “why” there is an emerging demand from researches around the world to explore natural, biodegradable and ecological solutions as potent alternatives to conventional antibiotics.

In a “how” perspective, the present study reflects the experimental design of CS-based systems to encapsulate TA. This compound as a polyphenol is a successful example of an antioxidant, regenerative with inflammation control properties and surprising antimicrobial potential. Microsystems were prepared by spray dryer technique and a satisfactory product yield of 32% when compared to reported values of similar microparticles produced by spray drying. Diameters obtained were around 7.4 µm, exhibited a spherical morphology with concavities on the outer surface, while hydrated microparticles were regular shape and smooth surface with some agglomeration tendency. Thermal stability of the particles as well as its substrates were proven, which allows to conclude that inlet temperature of spray drying do not affect their physical and chemical properties. No covalent bonds were found between TA and CS, according to the obtained results of DSC and FTIR. The adopted microencapsulation process also demonstrated a high encapsulation efficiency (98.50%).

Regarding biological properties, TA encapsulated showed significant lower values of antioxidant activity, compared to free TA free in solution due to its encapsulation system. Nevertheless, CMTA still has optimal antioxidant activity performance, plus protecting TA from degradation and providing biological stability, and controlled released (~0.8%). The developed microsystems were also bactericide against *S. aureus* ATCC as well as methicillin resistant to *S. aureus*, the predominant microorganism present in diabetic wounds. However, although its greats potential for wound healing, SS was not a gain to improve the microparticles antioxidant potential or either antimicrobial of microparticles properties.

To conclude, spray drying revealed to be an efficient microencapsulation process of CMTA. Bearing in mind obtained results, it is believed that devised CS microparticles have a great potential for TA wound delivery. At the end, it was possible to develop an efficient antimicrobial and antioxidant powder composed by natural compounds. However, further and more extensively studies should be performed in order to get a better characterization of the microparticles, to understand if TA has antimicrobial potential against other bacteria, and, to better understand and to obtain a more controlled and sustained release system for wound delivery.

5.2. Limitations and future prospects

Some limitations arose in the development of the present work, which forced an experimental alignment. The viscosity of CS revealed to be a limitation during the spray drying process, since the adherence of CS to the drying chamber was observed, which might compromise the final product yield, and the atomization process. In this sense, modified CS could also be an option, since its solubility in water without acetic addition, and /or other interesting bioactive polymers, like alginate or pectin. Pectin can be considered an alternative as a drug carrier for encapsulation phenolic extracts, like TA, due to antibacterial and antioxidant activity as well as its use as a carrier for drug delivery is already assessed (176). Besides, the combination of pectin and alginate, to produce microparticles is considered more efficient than pectin alone (177). Therefore, pectin with alginate could be an efficient alternative as drug carriers for TA encapsulation by spray drying.

In the same way, it would be interesting to test the Mini Spray Dryer B-290, a laboratory scale spray dryer more advanced that the spray dryer used, in attempt to obtain a higher product yield and, consequently, to minimize powder losses. Other range of TA concentrations could be tested in order to predict the antimicrobial potential against other bacteria, in particular *E. faecalis*, *P. aeruginosa* and *E. coli.* Another alternative to increase the spectrum of antimicrobial activity could be the incorporation of an adjuvant, a natural compound, with antimicrobial properties, in attempt to create a potential synergistic effect between its antimicrobial potential and the activity of TA. Besides that, antimicrobial activity of powdered CS microparticles should also be studied in order to predict their potential, in particular antimicrobial potential.

Although results of performed tests prove the potentials of developed microparticles, more studies should be done to guarantee the success of the incorporation of TA for a possible formulation to chronic wound application. Some characteristic of microparticles produced by spray drying, such as morphology, AE, and profile of TA release, depends on certain parameters of spray dryer, as well as the release medium and its pH. Therefore, different values of these parameters and conditions should be studied to improve some characteristics of the microparticles and optimize the microencapsulation process and/or bioactivity. Besides to the need of further physicochemical testing, cell culture experiments should be considered using i*n vitro* wound healing skin models to evaluate cell mobility and healing capacity in an advanced stage.

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Appendix

A. Antioxidant activity: calibration curve

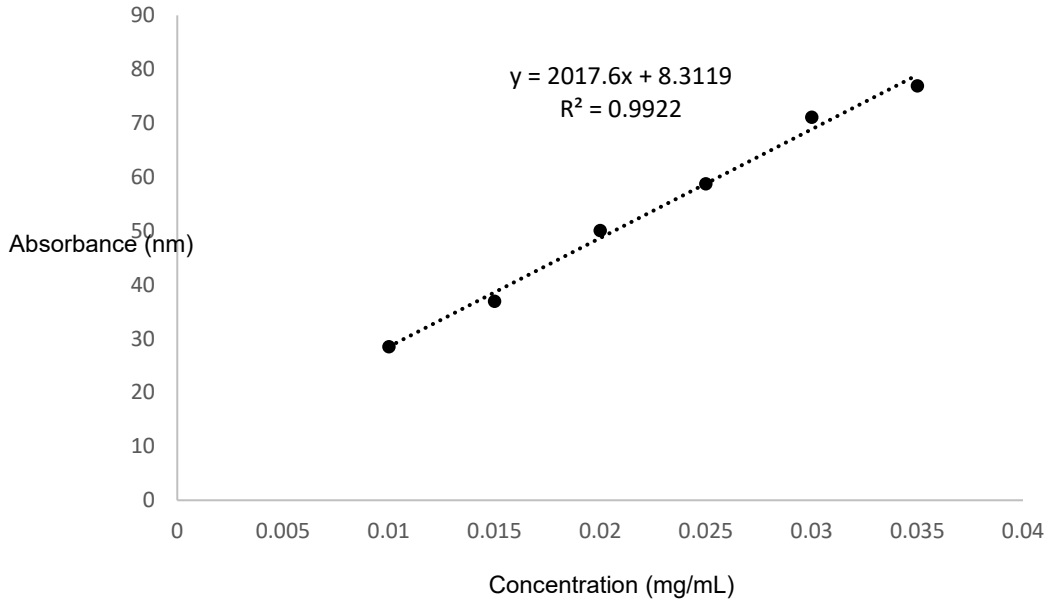

Figure A – Calibration curve for antioxidant activity estimated by ABTS method

B. High Performed Liquid Chromatography: calibration curve

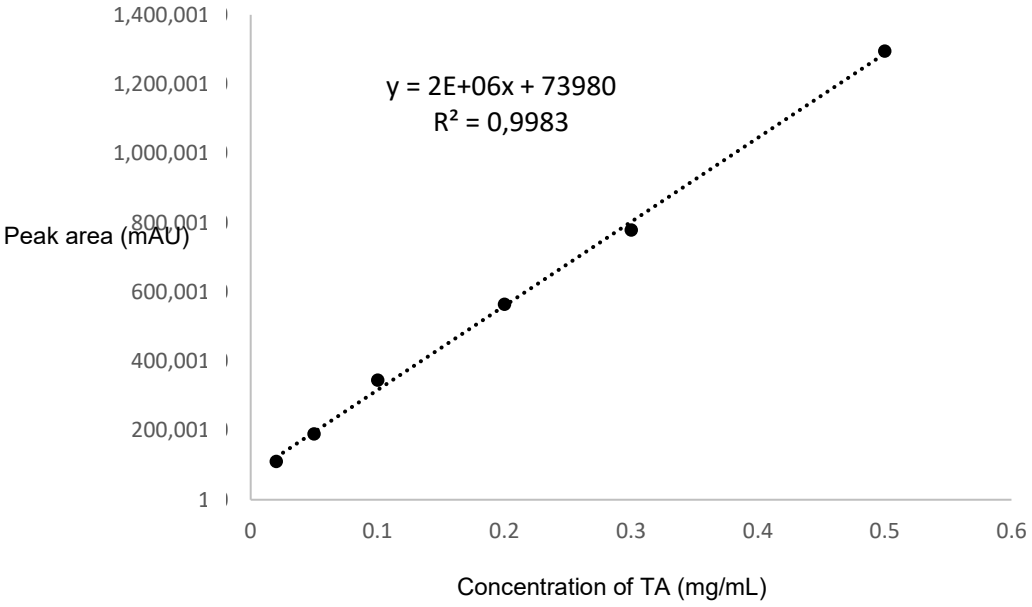

Figure B - Calibration curve of TA quantification in aqueous solutions by HPLC (0.02, 0.05, 0.1, 0.2, 0.3, and 0.5 mg/mL)