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WALTER DE SOUZA TAVARES

Desenvolvimento e caracterização de filmes de quitosana e zeina contendo ácido elágico para aplicações biomédicas

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SÍMBOLOS, SIGLAS E ABREVIATURAS

ANOVA	Análise de variância
ATCC	American Type Culture Colletion
BHI	Caldo cérebro-coração (do inglês: Brain Heart Infusion)
CFU	Unidades Formadoras de Colônia (do inglês: colony forming units)
*Ch*C	Clostridium histolyticum
CHI	Quitosana (do inglês: chitosan)
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetros
DPPH	2,2-diphenyl-1-picryl-hydrazila
EA	Ácido elágico (do inglês: ellagic acid)
FALGPA	N- [3- (2-furyl) acryloyl] -Leu-Gly-Pro-Ala
FTIR	Espectroscopia de Infravermelho com transformada de Fourier (do inglês: Fourier transform-infrared spectroscopy)
h	Hora (s)
IC_{50}	Concentração inibitória média de 50%
kV	Quilovolt
MBC	Concentração bactérida mínima (do inglês: Minimum bactericidal concentration)
mg/ml	Miligrama por mililitro.
ml	Mililitro

MIC	Concentração inibitória mínima (do inglês: Minimum Inhibitory Concentration)
mM	Milimolar
MPa	Megapascal
NCCLS	National Committee for Clinical Laboratory Standards
N	Normal
NMR	Ressonância Magnética Nuclear (do inglês: Nuclear magnetic resonance)
PBS	Tampão fosfato-salino (do inglês: Phosphate buffered saline)
SEM	Microscopia Eletrônica de Varredura (do inglês: Scanning electronic microscopy)
STD	Diferença na saturação de transferência (do inglês: saturation transfer difference)
U/ml	Unidade por mililitro
UV-VIS	Ultravioleta vísivel
v/v	Volume por volume
w/v	Peso por volume (do inglês: weigth/volume)
ºC	Grau (s) Celsius
µg	Micrograma
µg/ml	Micrograma por mililitro
µl	microlitro
λ	Comprimento de onda

Desenvolvimento e caracterização de filmes de quitosana e zeina contendo ácido elágico para aplicações biomédicas

RESUMO

Introdução: A quitosana (CHI) é um polímero natural biocompatível, biodegradável, biofuncional com efeitos antimicrobiano e antioxidante. A zeina é uma proteína com capacidade de carrear diferentes moléculas de interesse terapêutico. O ácido elágico (EA) é um polifenol que possui algumas atividades de interesse farmacêutico, dentre as quais, antimicrobiana e antioxidante. Diante disso, buscou-se avaliar a sua funcionalidade para aplicações biomédicas. **Objetivo:** Avaliar o potencial biofarmacêutico do ácido elágico, quitosana e zeina na composição de filmes para aplicações biomédicas. **Metodologia:** A atividade antimicrobiana foi determinada pelo método de microdiluição através da determinação da concentração inibitória mínima (MIC) e concentração bactericida mínima (MBC) A atividade antioxidante foi estudada pelo método de eliminação de radicais DPPH livres. As possíveis interações fármaco-polímeros foram avaliadas através de espectroscopia de RMN. As atividades anticolagenase e antielastase foram avaliadas por testes colorimétricos. Um desenho composto central foi usado para avaliar os efeitos de diferentes concentrações dos polímeros sobre variáveis-resposta dos filmes obtidos. A morfologia e possíveis alterações químicas dos filmes foram avaliadas por MEV e FTIR. Foi determinado ainda o módulo de elasticidade e a resistência dos filmes com melhor desempenho. **Resultados e discussões:** O EA apresentou atividade antimicrobiana frente à *Staphylococcus aureus* e *Pseudomonas aeruginosa,* e uma IC_{50} de 0,079 mg/ml em relação a sua atividade antioxidante. A zeina aumentou a atividade inibitória do EA frente *P.aeruginosa* e melhorou sua atividade antioxidante, demonstrando, ainda, atividade antimicrobiana e antioxidante isoladamente. A CHI potencializou a atividade inibitória do EA em ambas as bactérias e apresentou atividade antimicrobiana mais discreta. O espectro de RMN obtido revelou interação entre EA com zeina. Os filmes obtidos apresentaram morfologia e propriedades biofuncionais e mecânicas satisfatórias. **Conclusões:** A associação de EA com CHI-zeina mostrou-se favorável a partir da melhoria nas propriedades biológicas do biativo. Os filmes obtidos a partir desta associação apresentaram características favoráveis para seu uso em aplicações biomédicas.

Palavras-Chave: Quitosana; Zeina; Ácido elágico; Filmes poliméricos.

Development and characterization of chitosan and zein films containing ellagic acid for biomedical applications

ABSTRACT

Introduction: Chitosan (CHI) is a biocompatible, biodegradable, biofunctional natural polymer with antimicrobial and antioxidant effects. Zein is a protein that has the ability to carry different molecules of therapeutic interest. Ellagic acid (EA) is a polyphenol that has some activities of pharmaceutical interest, such as antimicrobial and antioxidant action. In view of these, it was aimed to evaluate its functionality for biomedical applications. **Objective**: To evaluate the biopharmaceutical potential of ellagic acid, chitosan and zein in the films composition for biomedical applications. **Methodology**: The antimicrobial activity was determined by the microdilution method through the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The antioxidant activity was studied using the DPPH free radical scavenging method. The possible drug-polymer interactions were evaluated by NMR spectroscopy. The anti-collagenase and antielastase activities were determined using colorimetric tests A central composite design was used to evaluate the effects of different polymer concentrations on the response variables of the films obtained. The morphology and possible chemical changes of the films were studied by SEM and FTIR. It was also determined the modulus of elasticity and the strength of films with better performance. **Results and discussion**: EA presented antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and an IC_{50} of 0.079 mg/ml regarding its antioxidant activity. Zein improved the inhibitory activity of EA against *P.aeruginosa* and improved its antioxidant activity, demonstrating antimicrobial and antioxidant activity itself. CHI enhanced the inhibitory activity of EA against both bacteria and also showed itself a more discrete antimicrobial activity. NMR spectroscopy revealed the interaction between EA with zein. The films obtained presented satisfactory morphology and biofunctional and mechanical properties. **Conclusions:** The association of EA with CHI-zein has shown to be favorable, considering the biological aspects studied. The obtained films from this association showed favorable characteristics for their use in biomedical applications.

Keywords: Chitosan; Zein Ellagic acid; Polymeric films

1 INTRODUÇÃO

A pesquisa de tecnologias aplicadas à saúde tem apresentado grandes avanços nos últimos anos, a partir da aplicabilidade e funcionalidade de novos produtos e/ou melhoria dos já existentes.

Os polímeros tornaram-se valiosas ferramentas terapêuticas, visto que podem ser utilizados sob diferentes formas em aplicações farmacêuticas, tais como: revestimento de formas sólidas, nanoparticulas, curativos, hidrogeis, *drug delivery*, dentre outros.

Atualmente, os sistemas poliméricos de liberação controlada vêm sendo amplamente estudados, uma vez que representam uma estratégia muito útil para a incorporação de fármacos, objetivando um controle na liberação ou direcionamento do fármaco a alvos específicos (BIZERRA; SILVA, 2016).

Um dos polímeros naturais mais extensivamente estudados neste âmbito é a quitosana (CHI), obtido a partir da desacetilação da quitina, encontrada nas carapaças de crustáceos. Este polissacarídeo é biocompativel, biodegradável, apresenta baixa toxicidade, não antigênico, biofuncional e apresenta grande potencial tecnológico. A CHI apresenta algumas propriedades biológicas já descritas que despertam grande interesse farmacêutico, dentre as quais: analgesia (OKAMOTO et al., 2002), atividade antimicrobiana (HELANDER et al., 2001), atividade antioxidante (XING et al., 2005), atividade hemostática (OKAMOTO et al., 2003) e atividade cicatrizante (KIM et al., 2015). A mesma já foi utilizada sob diversas formas: filmes, hidrogeis, nanofibras, nanoemulsões, dentre outras. A sua associação com diferentes substâncias orgânicas e inorgânicas, dentre elas a zeina e o ácido elágico (ESCAMILLA-GARCIA et al., 2013; GOPALAKRISHNAN et al., 2014), já fora descrita na literatura.

A zeína é uma proteína derivada do milho, que vêm sendo recentemente utilizada no revestimento de formas sólidas e na composição em sistemas de liberação de fármacos. Este biopolímero já demonstrou a capacidade de carrear diferentes moléculas de interesse terapêutico, capacidade filmogênica, biodegrabilidade e biocompatibilidade. Nos achados de Soliman et al. (2014), apresentou atividade antimicrobiana e antioxidante. Neste sentido, a zeina apresenta-se funcional e biologicamente favorável para sua utilização em tecnologias aplicadas a fármacos (TANG; ZHUANG, 2014).

O ácido elágico (EA) é um polifenol, encontrado em frutos de diferentes espécies como o morango e a romã, tendo-lhe sido atribuídas algumas atividades biológicas como: atividade antimicrobiana e antioxidante (NAYEEM; KARVEKAR, 2011), assim como atividade cicatrizante (AL-OBAIDI et al., 2014). Além destas, são relatadas algumas outras atividades do EA: efeito hepatoprotetor (GARCÍA-NIÑO; ZAZUETA, 2015), atividade neuroprotetora (OLIVEIRA, 2016), ação antihemorrágica (GOPALAKRISHNAN et al., 2014), ação antiulcerogênica e gastroprotetora (BESERRA et al., 2011; CHATTERJEE et al., 2012).

Diante das propriedades biológicas promissoras do EA e das características dos polímeros CHI e zeina, o presente estudo buscou aprofundar este conhecimento, utilizando as atividades antimicrobiana, antioxidante e antienzimática para avaliar as propriedades biológicas destas substâncias isoladas ou em associação. A partir destes achados, foram desenvolvidos filmes poliméricos com vistas a sua utilização no tratamento de lesões cutâneas e/ou em outras condições biomédicas nas quais as propriedades farmacológicas do EA possam ser favoráveis.

2 OBJETIVOS

2.1 GERAL

Avaliar o potencial biofarmacêutico de ácido elágico, quitosana e zeina para a composição de filmes para aplicações biomédicas.

2.2 ESPECÍFICOS

a) Determinar a atividade antimicrobiana e antioxidante *in vitro* do ácido elágico, da quitosana e da zeina isolados e em combinação.

b) Avaliar o potencial de inibição das enzimas colagenase e elastase pelo ácido elágico.

c) Verificar as possíveis interações químicas entre ácido elágico, quitosana e zeina através de Ressonância Magnética Nuclear.

d) Desenvolver e caracterizar química, física e biofarmacêuticamente os filmes poliméricos a base de quitosana e zeina contendo ácido elágico.

3 CAPÍTULO 1

CHITOSAN FILMS: A PROMISING TECHNOLOGY FOR WOUND HEALING

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Chitosan films: a promising technology for wound healing

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Abstract

Chitosan is a polysacaride based biomaterial that presents favorable characteristics to be used in wounds treatment, especially when associated with other substances in the form of films. It has already demonstrated some biological activities such as analgesic, antimicrobial, antioxidant, hemostatic and healing. Chitosan films show high tensile strength, good flexibility, high sorption, low solubility, drug loading and controlled release abilities. Several papers have demonstrated the importance of this biomaterial isolately or in combination with other substances on promoting wound healing. Therefore, the present mini-review attemps to highlight the different characteristics, developments and aplicabilities of chitosan in film formation for wound healing.

Keywords: Chitosan, film, wound, healing, drug delivery system

1. Introduction

Chitosan (CHI) is a natural polysaccharide, obtained from the deacetylation process of chitin, found mainly in the shells of crustacenas. It is a biodegradable, biocompatible, non-toxic, non-antigenic, biofunctional polymer that has recently gained strong attention regarding its pharmaceutical applications[1,2], among those the treatment of wounds[3]. Figure 1 illustrates the basic monomeric structure of CHI.

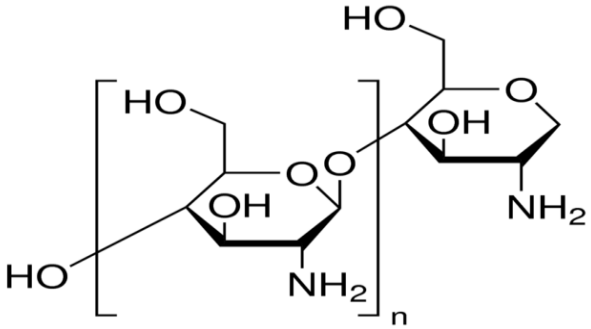

Figure 1 – Chemical structure of a monomer of chitosan.

Concerning healing, CHI has been investigated in different pharmaceutical forms, such as: membranes[4,5], hydrogels[6], nanofibres[7] and nanoemulsions[8]. Recently CHI was studied in the film form as a healing adjuvant, demonstrating the ability to stimulate the proliferation of keratinocytes, resulting in epidermal regeneration with optimal biocompatibility[9], and to promote macrophages activation and trigger collagen production[10].

Therefore, CHI is an important raw material to produce films for different biomedical applications, mainly due to its bioavailability, biocompatibility, biodegradability, manufacturing, low cost and mainly due to its biopharmaceutics properties that favor its use in the pharmaceutical field.

2. Biological properties of chitosan

CHI presents some relevant properties to assist the healing process. Some biological properties that have been demonstrated are shown in table 1.

Table 1. Biologial properties attributed to chitosan

Biological activity	Expected effect
Analgesia[11]	CHI and also chitin can reduce the effects of bradykinin, leading to a reduction on the pain intensity.
Antimicrobial[12,13]	CHI permeates the membrane of some Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli*), causing bacteriostatic effect, and has bactericidal activity against some gram-positive bacteria.
Antioxidant[14,15]	The antioxidant activity of CHI is related to the degree of deacetylation, the higher is this level; the more intense will be this effect.
Hemostatic [15]	CHI promotes an increment in platelet aggregation and reduces the coagulation time.
Healing[9,10]	CHI has reduced the healing time of superficial wounds.

In the view of these biological properties, CHI may be associated with other substances (drugs of not) in order to improve its therapeutic potential, such as in the process of skin and/or connective tissue regeneration.

3. The physical properties of chitosan films

The most common method for obtaining CHI films is solvent evaporation, also known as casting. The preparation process (Figure 2) consists on the formation of CHI solutions in an acidic medium, usually acetic and more uncommonly chloride acid, which is homogeneized, poured into petri dishes and stored for solvent evaporation, which may occur at room temperature or in an oven with temperatures up to 50º C[1,17-19].

During the preparation, other substances (adjuvants, such as plasticizers or active substances) can be associated to improve the film mechanical and/or biological properties.

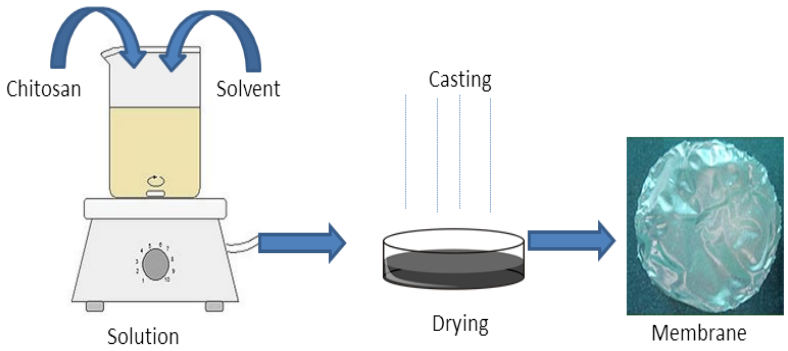

Figure 2 – Casting method used to prepare chitosan films.

CHI films have suitable characteristics to favour the wound healing, such as in curatives: high tensile strength, good flexibility/elasticity, adequate thickness and smoothness of the film surface, high bioadhesiveness and water sorption capacity[20,21].

The requisites for a good wound dressing are: easy aplication, barrier properties (dependent on thickness, porosity and density), good bioadhesiveness, high flexibility, high sorption (keeping the environment with adequate moisture), low solubility, good elasticity, biodegradability, resistence to low tensions, associated with biological properties that could contribute to the healing process[22]. Therefore, the physical and biological properties of CHI are relevant elements for the development of technologies regarding the healing promotion.

4. Chitosan films and healing

In the pharmaceutical industry there are some formulations based on chitosan used for healing, in the form of films, spray and gel. Chitosan antibacterial spray (Hernan Huibo Medical CO, China) and the silver-health gel containing the association chitosan-iron (Hernan Huibo Medical CO, China) are specialities used to treat burns, pressure lesions, surgical and infected wounds, such as furuncules. CHI has become attractive to this field, first due to its high biocompatibility with human cells, and secondarily due to its ability to interact with organic and inorganic substances[9,23] to promote their controlled release. ChitoGauze PRO® and ChitoFlex PRO® (Tricol Biomedical Incorporated, USA), Axiostat® (Axio Biosolutions Private, India) and Chitosan Biomedical Dressing® (Hangzhou Qiandao Lake Longer Biotechnology CO, china) are

chitosan coating, in the form of film and/or membranes used to contain hemorrhagies and to promote wound protection and antibacterial inhibition.

Despite the few products marketed, CHI films are still an appealing thematic and any further improvement in these technologies could open a new perspective for wound healing.

The physical properties of CHI are used in films that assist the controlled release of certain substances, whose pharmacological properties are known. In the composition of films, CHI has been associated with metals Cu, Zn and Mg[1], polyethylene[2], gelatine[3], chondroitin sulfate and zinc oxide[5], zein, salicylic acid and hyaluronic acid[7], silver nanoparticles[24], chlorhexidine diacetate[25], nitric oxide[26], calcium alginate[27], PVA and ibuprofen[28], caffeic acid and polycaprolactone[29], among others.

Such associations aim to improve the films characteristics, such as mechanical properties, sorption, solubility, thickness and antimicrobial activity, facilitating the cicatricial process. The healing potential of CHI in treating cutaneous lesions in rats has been demonstrating, by accelerating the wound contraction[30].

Table 2 shows some studies that associated CHI with other substances in order to obtain films for healing treatment.

Table 2. CHI associated with other substances in films.

Associated substance	**Observed effects**
Polyethylene[2]	Improved mechanical characteristics, such as increased tensile strength, transparency and high water absorption capacity. Also, enhanced its antimicrobial effect, qualifying it for biomedical applications where bacteremy is present, such as curatives.
Gelatin, chondroitin sulfate and zinc oxide[5]	Promoted an environment favorable to cell proliferation and as a result, skin regeneration and thus its healing.
Gelatin and tannic acid[31]	Improved physical properties, such as high tensile strength, good elasticity, high water uptake capacity in adittion to improve the healing

	process and antimicrobial activity.
Zein, hyaluronic and salicylic acids[7]	An alternative for treating wounds, as they presented sustainable release and biofunctional properties of relevant interest for biomedical applications, such as: mechanical properties compatible with the skin, optimal water vapor permeation, biodegradability, sustained release of salicylic acid, atoxicity, inhibitory activity against *Staphylococcus aureus*, fibroblastic migration, adhesion and proliferation.
Sagu and silver nanoparticles[24]	Effective films to the cicatricial process, stimulating the fibroblastic migration to the wound bed with a consequent increment in collagen production. Nonetheless, the physical properties of the film were impaired, once the water sorption capacity and tensile strength were reduced.
Chlorhexidine diacetate[25]	Promoted the drug controlled release, thus reducing its intrinsic cytotoxicity.
Nitric oxid[26]	Films with a controlled and satisfactory release in the wound bed, accelerating the wound re-epithelialization with a reduction in the infiltration of inflammatory cells. The physical properties of these films remained ideal both in terms of tensile strength and their water absorption capacity.
PVA and ibuprofen[28]	Controlled release of ibuprofen to act on wound healing. Such association showed an adequate and sustainable release profile of ibuprofen that has supported the inflammatory reduction on the wound.
Extract from barks of *Mimosa tenuiflora*[32]	Films with a high proportion of *M. tenuiflora* extract showed an increment in water absorption and improved the antimicrobial activity against Gram-negative and positive bacteria.

The use of CHI in the form of films associated with other substances has been well evidenced, according to the aforementioned examples. This biopolymer has gained great interest in the last decades as a promising substance for new technologies aimed to treat wounds, regarding its physical and biological properties. Accordingly, CHI presents a great potential to be explored in the pharmaceutical industry in several ways, among those the composition of films.

5. Conclusions

CHI is a natural polymer with antimicrobial and healing properties well described in the literature. It presents physical characteristics that allow its usage in wound treatment technologies, such as high flexibility, tensile strength and water absorption capacity. Aditionally, it presents ideal characteristics to compose drug release systems. Its biopharmaceutical characteristics, such as antimicrobial, antioxidant and antihemorragic are also very beneficial, as it could also support the healing process. In summary, it can be stated that CHI can be used for wounds treatments, especially in the form of films, associated with other biopolymers and/or drugs. These membranes are currently among the most promising devices regarding a more effectient wound healing.

6. References

1. Cardoso, M. J. B., Barbosa, R. C., Ries, A., Fidelis, T., & Fook, M. V. L. (2012). Ensaios físico-químicos e mecânicos de membranas de quitosa com cobre, zinco e magnésio. *Revista Eletronica de Materiais e Processos*, 1, 1-6. Retrieved from http://www2.ufcg.edu.br/revista-remap/index.php/REMAP/article/viewFile/240/229

2. Doulabi, A. H., Mirzadeh, H., Imani, M., & Samadi, N. (2013). Chitosan/ polyethylene glycol fumarate blend film: physical and antibacterial properties. *Carbohydrate Polymers*, 92(1). 48-56. doi: 10.1016/j.carbpol.2012.09.002

3. Shahram, E., Sadraie, S. H., Kaka, G., Khoshmohabat, H., Hosseinalipour, M., Panahi, F., & Naimi-Jamal, M. R. (2013). Evaluation of chitosan-gelatin films for use as postoperative adhesion barrier in rat cecum model. *International Journal of Surgery*,11(10), 1097-1102. doi: 10.1016/j.ijsu.2013.09.012

4. Hirose, K., Onishi, H., Sasatsu, M., Takeshita, K., Kouzuma, K., Isowa, K., & Machida, Y. (2007). *In vivo* evaluation of Kumazasa extract and chitosan films containing the extract against

deep skin ulcer model in rats. *Biological and Pharmaceutical Bulletin*, 30(12), 2406-2411. doi: 10.1248/bpb.30.2406

5. Cahú, T. B., Silva, R. A., Silva, R. P. F., Silva, M. M., Arruda, I. R. S., Silva, J. F., Costa, R. M. P. B., Santos, S. D., Nader, H. b., & Bezerra, R. S. (2017). Evaluation of chitosan-based films containing gelatin, condroitin 4-sulfate and ZnO for wound healing. *Applied Biochemistry and Biotechnology*, 183(3), 765-777. doi: 10.1007/s12010-017-2462-z.

6. Chen, X., Zhang, M., Wang, X., Chen, Y., Yan, Y., Zhang, L., & Zhang, L. (2017). Peptide-modified chitosan hydrogels promote skin wound healing by enhancing wound angiogenesis and inhibiting inflammation. *American Journal of Translational Research*, 9(5), 2352-2362. doi: 10.1007/s12010-017-2462-z.

7. Figueira, D. R., Miguel, S. P., Sá, K. D., & Correia, I. J. (2016). Production and characterization of polycaprolactone-hyaluronic acid/chitosan-zein electrospun bilayer nanofibrous membrane for tissue regeneration. *International Journal of Biological Macromolecules*, 93, 1100-1110. doi: 10.1016/j.ijbiomac.2016.09.080

8. Thomas, L., Zakir, F., Mirza, M. A., Anwer M. K., Ahmad, F.J., & Iqbal, Z. (2017). Development of Curcumin loaded chitosan polymer based nanoemulsion gel: *In vitro, ex vivo* evaluation and *in vivo* wound healing studies. *International Journal of Biological Macromolecules*, 101, 569-579. doi: 10.1016/j.ijbiomac.2017.03.066

9. Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan – A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, 36(8), 981-1014. doi: 10.1016/j.progpolymsci.2011.02.001

10. Silva, H. S. R. C., Santos, K. S. C. R., & Ferreira, E. I. (2006). Quitosana: derivados hidrossolúveis, aplicações farmacêuticas e avanços. *Química Nova*, 29(4), 776-785. doi: 10.1590/S0100-40422006000400026.

11. Okamoto, Y., Kawakami, K., Miyatake, K., Morimoto, M., Shigemasa, Y., & Minami, S. (2002). Analgesic effects of chitin and chitosan. *Carbohydrate Polymers*, 49, 249-252. doi: 10.1016/S0144-8617(01)00316-2

12. Helander, I. M., Nurmiaho-Lassila, E. L., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology*, 71(2-3), 235-244. doi: 10.1016/S0168-1605(01)00609-2.

13. Goy, R. C., Morais, S. T. B., & Assis, O. B. G. 2016. Evaluation of the antimicrobial activity of chitosan and its quaternized derivate on *E.coli* and *S.aureus* growth. *Brazilian Journal of Pharmacognosy*, 26, 122-127. doi: 10.1016/j.bjp.2015.09.010.

14. Park, P. J., Je, J. Y., & Kim, S. K. (2004). Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. *Carbohydrate Polym*ers, 55(1), 17-22. doi: 10.1016/j.carbpol.2003.05.002.

15. Xing, R., Liu, S., Guo, Z., Yu, H., Wang, P., Li, C., li, Z., & Li, P. (2005). Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities *in vitro*. *Bioorganic & Medicinal Chemistry*, 13(5), 1573-1377. doi: 10.1016/j.bmc.2004.12.022.

16. Okamoto, Y., Yano, R., Miyatake, K., Tomohiro, I., Shigenasa, Y., & Minami, S. (2003). Effects of chitin and chitosan on blood coagulation. *Carbohydrate Polym*ers, 53(3), 337-342. doi: 10.1016/S0144-8617(03)00076-6.

17. Barreto, R. S. S., Quintans, J. S. S., Barreto, A. S., Albuquerque-Júnior, R. L. C., Galvão, J. G., Gonsalves, J. K. M. C., Nunes, R. S., Camargo, E. A., Lucca-Júnior, W., Soares, R. c., Feitosa, V. L. C., & Quintans-Júnior, L. J. (2014). Improvement of wound tissue repair by chitosan films containing (–)-borneol, a bicyclic monoterpene alcohol, in rats. *International wound journal*, 13(5), 799-808. doi: 10.1111/iwj.12385.

18. Martins, E. A., Invernizzi, M. S., Campos, M. G. N., Teodoro, P. A., Contieri, M. B., & Silva, L. C. L. C. (2013). Emprego de membrana de quitosana em feridas cutâneas induzidas experimentalmente em equinos. *Ciência Rural*, 43(10), 1824-1830. doi: 10.1590/S0103-84782013001000015.

19. Aldana, A. A., Barrios, B., Strumia, M., Correa, S., & Martinelli, M. (2016). Dendronization of chitosan films: surface characterization and biological activity. *Reactive and Functional Polymers*, 100, 18-25. doi: 10.1016/j.reactfunctpolym.2016.01.003.

20. Khan, T. A., Peh, K. K., & Ch'ng, H. S. (2000). Mechanical, bioadhesive strength and biological evaluations of chitosan films for wound dressing. *Journal of Pharmacy and Pharmaceutical Science*, 3(3), 303-311. Retrieved from: https://sites.ualberta.ca/~csps/JPPS3(3)/K.Peh/Peh.pdf

21. Escamilla-García, M., Calderón-Domínguez, G., Chanona-Pérez, J. J., Farrera-Rebollo, R. R., Andraca-Adame, J. Á., Arzate-Vázquez, I., Mendez-Mendez, J. V., & Moreno-Ruiz, L. A. (2013). Physical and structural characterisation of zein and chitosan edible films using nanotechnology tools. *International Journal of Biological Macromolecules*, 61, 196-203. doi: 10.1016/j.ijbiomac.2013.06.051.

22. Kumar, R. S. (2014). *Textiles for industrial applications*. New York: CRC Press – Taylor e Francis group.

23. Macêdo, M. O. R., Macêdo, H. R. A., Santos, Z. M., Pereira, M. R., & Alves Jr, A. (2010). Avaliação da modificação de membranas de quitosana tratadas por plasma de hidrogênio para aplicações biomédicas. *Revista Brasileira de Aplicações de Vácuo*, 29(1), 31-36. doi: 10.17563/rbav.v29i1-2.850.

24. Arockianathan, P. M., Sekar, S., Kumaran, B., & Sastry, T. P. (2012). Preparation, characterization and evaluation of biocomposite films containing chitosan and sago starch impregnated with silver nanoparticles. *International Journal of Biological Macromolecules*, 50(4), 939-946. doi: 10.1016/j.ijbiomac.2012.02.022.

25. Ambrogi, V., Pietrella, D., Nocchetti, M., Casagrande, S., Moretti, V., Marco, S., & Ricci, M. (2017). Montmorillonite-chitosan-chlorhexidine composite films with antibiofilm activity and improved cytotoxicity for wound dressing. *Journal of Colloid and Interface Science*, 491(1), 265-272. doi: 10.1016/j.jcis.2016.12.058.

26. Kim, J. O., Noh, J. K., Thapa, R. K., Hasan, N., Choi, M., Kim, J.H., Lee, J. H., Ku, S. K., & Yoo, J. W. (2015). Nitric oxide-releasing chitosan film for enhanced antibacterial and *in vivo*

wound-healing efficacy. *International Journal of Biological Macromolecules*, 79, 217-225. doi: 10.1016/j.ijbiomac.2015.04.073.

27. Kong, Y., Xu, R., Darabi, M. A., Zhong, W., Luo, G., Xing, M. M. Q., & Wu, J. (2016). Fast and safe fabrication of a free-standing chitosan/alginate nanomembrane to promote stem cell delivery and wound healing. *International Journal of Nanomedicine*, 11, 2543-2555. doi: 10.2147/IJN.S102861.

28. Morgado, P. I., Miguel, S. P., Correia, I. J., & Ricardo, A. A. (2016). Ibuprofen loaded PVA/chitosan membranes: a highly eficiente strategy towards na improve skin wound healing. *Carbohydrate Polymers*, 159, 136-145. doi: 10.1016/j.carbpol.2016.12.029.

29. Oh, G. W., Ko, S. C., Je, J. Y., Kim, Y. M., Oh, J.H., & Jung, W.K. (2016). Fabrication, characterization and determination of biological activities of poly (ε-caprolactone)/chitosan-caffeic acid composite fibrous mat for wound dressing application. *International Journal of Biological Macromolecules*, 93, 1549-1558. doi: 10.1016/j.ijbiomac.2016.06.065.

30. Fráguas, R. M., Rocha, D. A., Queiroz, E. R., Abreu, C. M. P., Sousa, R. V., & Oliveira Júnior, E. M. (2015). Caracterização química e efeito cicatrizante de quitosana com baixos valores de massa molar e grau de acetilação em lesões cutâneas. *Polimeros*, 25(2), 205-211. doi: 10.1590/0104-1428.1778.

31. Lu, B., Wang, T., Li, Z., Dai, F., Lv, L., Tang, F., Yu, K., Liu, J., & Lan, G. (2016). Healing of skin wounds with a chitosan-gelatin sponge loaded with tannins and platelet-rich plasma. *International Journal of Biological Macromolecules*, 82, 884-891. doi: 10.1016/j.ijbiomac.2015.11.009.

32. Valencia-Gómez, L. E., Martel-Estrada, S. A., Vargas-Requena, C., Rivera-Armenta, J. L., Alba-Baena, N., Rodrígues-Gonzáles, C., & Olivas-Armendáriz, I. (2016). Chitosan/*Mimosa tenuiflora* films as potencial cellular patch for skin regeneration. *International Journal of Biological Macromolecules*, 93, 1217-1225. doi: 10.1016/j.ijbiomac.2016.09.083.

4 CAPÍTULO 2

BIOPHARMACEUTICAL ACTIVITIES RELATED TO ELLAGIC ACID, CHITOSAN AND ZEIN AND THEIR IMPROVEMENT BY ASSOCIATION

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Biopharmaceutical activities related to ellagic acid, chitosan and zein and their improvement by association

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Abstract

Ellagic acid (EA) has demonstrated several biological properties, such as antioxidant, antimicrobial and enzymatic inhibition. Zein and chitosan (CHI) are natural polymers whose biological pontential have also been explored. Therefore, this paper aimed to evaluate the antimicrobial, antioxidant, anti-collagenase and antielastase properties of ellagic acid, zein and chitosan isolated and in combination. The antimicrobial activity was determined following the microdilution method to assess the minimum inhibitory and bactericide concentrations. The antioxidant activity was determined using the DPPH free radical scavenging method. The anticollagenase and antielastase activities were evaluated by specific colorimetric tests. EA has shown inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* together with an IC_{50} of 0.079 mg/ml regarding its antioxidant activity. EA also showed significant collagenase and elastase inhibition. Zein improved the antimicrobial activity of EA against *P. aeruginosa* and has also been able to improve the antioxidant activity of EA when both were combined. It has also shown antimicrobial and antioxidant activities itself. CHI enhanced the inhibitory activity of EA against both strains and it showed itself an acceptable antimicrobial activity. NMR experiment confirmed the interaction between EA and zein, which could explain the improvement on its biological performance, what has not been noticeable with CHI.

Key–words: Ellagic acid. Zein. Chitosan. Antioxidant. Antimicrobial. Antienzimatic

1. Introduction

Ellagic acid (EA) is a phenolic compound belonging to the group of ellagitannins, found in certain fruits such as strawberries and pomegranates. Some biological activities are associated to this molecule, such as: antimicrobial against *Staphylococcus aureus* (Rúa et al., 2010), *Pseudomonas aeruginosa* (Jayaraman et al., 2010), *Escherichia coli, Klebsiella pneumoniae* and *Bacillus subtilis* (Nayeem, Karvekar, 2011), antioxidant (Nayeem, Karvekar, 2011), healing (Al-Obaidi et al., 2014) and anti-hemorrhagic (Gopalakrishan et al., 2014).

The antioxidant properties are related to its reducing characteristics, acting at the beginning and in the propagation stages of the oxidative process (Sousa et al., 2007). Being a phenolic compound, EA (Figure 1) presents structurally a high antioxidant activity (Nayeem, Karvekar, 2011). Substances with antioxidant activity have the ability to reach free radicals and stabilize or deactivate them before they attack biological targets in cells (Sousa et al., 2007).

Chitosan (CHI) is a natural polysaccharide, obtained from the deacetylation of chitin, found in crustacean shells. It is a biodegradable, biocompatible, non-toxic, non-antigenic and biofunctional polymer that has been widely investigated recently regarding its pharmaceutical applications (Cardoso et al., 2012; Doulabi et al., 2013). It has also demonstrated some biological activities, such as healing and antibacterial (Sharam et al., 2013; Doulabi et al., 2013).

Zein is a biodegradable and renewable plant protein found in the maize endosperm (*Zea mays*) and corresponds to 80% of the protein content in the corn seed (Corradini et al., 2014). In the pharmaceutical industry it has been used in the composition of nanoformulations, coating of solid forms and in the composition of drug delivery systems (Sousa et al., 2012a; Sousa et al., 2012b), where its controlled release ability has been demonstrated. The antioxidant activity of zein has been identified, but barely explored (Tang, Zhuang, 2014).

Collagenase is a meloproteinase responsible for cleaving collagen and elastin fibers, decreasing the skin resistance and elasticity (Thring, Hili, Naughton, 2009). Elastase is an enzyme from the chymotrypsin protease family of and may act on the cleavage of elastin, collagen, fibronectin and other extracellular matrix proteins (Thring, Hili, Naughton, 2009). Substances with antielastase activity prevent loss of skin elasticity. Pharmaceutical actives that have anti-collagenase and/or antielastase activity may help to maintain/recover the skin integrity and receive great interest in the pharmaceutical and cosmetic industries.

EA has been studied in association with chitosan, but there are few studies regarding its association with zein, and none enrolling both polymers. Thus, taking into account the properties of EA and zein and CHI polymers, the purpose of the present work has been to investigate the antimicrobial and antioxidant properties of ellagic acid, zein and chitosan isolated and in combination, evaluate the anti-collagenase and antielastase properties of EA and the possible

chemical interactions between EA, zein and CHI through ^{1}H Nuclear Magnetic Resonance (NMR), which could explain these outcomes.

2. Material and methods

2.1 Material

Ellagic acid (purity ≥95%), zein, chitosan (deacetylation degree = 85%, molecular weight = 1,9X10^{5} Da), *N*-succinyl-(Ala)3-*p*-nitroanilide, 2,2-diphenyl-1-picryl-hydrazila (DPPH) were purchased from Sigma-Aldrich (USA), collagenase activity kit was purchased from Abcam® (USA), Mueller Hinton and Brain Heart Infusion (BHI) broth have been purchased from Kasvi® (Brazil). Deuterated solvents D_2O 99.9%, CD_3OD 99.8% were purchased from Eurisotop (France). All the other reagents were analytical grade and used as received.

2.2 Antimicrobial activity assay

For the antimicrobial evaluation, an amount of EA: polymer 1:50 (w/w) has been dissolved in distilled water and used as the initial concentration for the tests. When the polymers were mixed, equal amounts of each have been used. EA has also been tested alone under the same conditions and used as a comparison group.

The American Type Culture Colletion (ATCC) - *Staphylococcus aureus* (ATCC 6538P) and *Pseudomonas aeruginosa* (ATCC 9027) were used as models, whose were maintained and cultured according to the specific conditions of National Committee for Clinical Laboratory Standards (NCCLS, 2006). The inoculum for the tests has been prepared by culturing the bacterial strains in BHI broth at 37± 1° C for 18 h. After culturing, the concentration was adjusted to 1.5x10^{8} CFU/ml (0.5 McFarland scale), in accordance with NCCLS (2006). The antibacterial activity of EA and polymers was achieved by determining the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) following the microdilution methodology in accordance with the CLSI guidelines (NCCLS, 2006; CLSI, 2006). To determine the effect, serial dilutions from the tested substances have been made in sterile BHI broth in a 96-well sterile plate. MIC was defined as the lowest concentration of the tested sample capable to completely inhibit the growth of the assayed bacteria. To determine the MBC an aliquot of 10 µl obtained from the three wells just above the MIC were seeded in Mueller-Hinton Agar and incubated for 48 h at 37 °C. MBC was defined as the minimum concentration required to eliminate most (≥99.9%) viable microorganisms after incubation. All assays were performed in triplicate under strictly aseptic conditions.

2.3. Antioxidant activity

The antioxidant activity was evaluated according to the methodology proposed by Sousa *et al.* (2007) based on the consumption of the radical DPPH.

For this assay, different concentrations of EA (250, 125, 75, 50 and 25 μg/ml^{-1}) and zein and CHI at 2.5, 2, 1.5, 1 and 0.5 mg/ml^{-1} in pure methanol were used. When the substances were assayed together, the concentrations of EA were combined to the concentration 2.5 mg/ml^{-1} of each polymer.

Aliquots of 0.3 ml of each tested solution were added to 2.7 ml of DPPH methanolic solution at 40 μg/ml^{-1} and kept in the dark. The absorbance of DPPH in methanol at the initial concentration was measured. The blank was prepared using 0.3 ml of each tested solution and 2.7 ml of pure methanol. After 30 minutes, the absorbance (Biospectro SP-22, Brazil) has been determined at the wavelength of λ= 517 nm. The assay was performed in triplicate and the antioxidant activity in terms of % was calculated according to the following equation:

$$AA\ (\%) = \frac{A_1 - A_2}{A_1} \times 100 \quad (1)$$

Where A_1 is the absorbance of the DPPH solution in methanol and A_2 is the absorbance of the tested solution after reacting with DPPH.

For the calculation of the IC_{50}, the concentration able to reduce 50% of DPPH radical, a linear fitting model has been used, adjusting the linear value (*y)* to 50.

2.4. Anticolagenase activity assay

The anticollagenase activity was performed based on the methodology proposed by Thring, Hili and Naughton (2009), where 25 μg of pure EA dissolved in sterile ultra-pure Milli-Q water has been used. Collagenase from *Clostridium histolyticum* (*Ch*C) was dissolved in tricine buffer (50 mM, pH 8.0) immediately prior to use at an initial concentration of 0.35 U/ ml. The synthetic substrate N- [3- (2-furyl) acryloyl] -Leu-Gly-Pro-Ala (FALGPA) (40 μl) was dissolved in the buffer (60 μl) according to the supplier information. EA was incubated with the enzyme for 15 minutes prior to substrate addition. The final reaction mixture contained tricine buffer, 0.8 mM FALGPA, 0.1 unit of *Ch*C and 25 μg of EA. Negative controls were performed with ultrapure Milli-Q water. The absorbance at λ=345 nm was measured immediately after the substrate incorporation and continuously for 20 minutes in kinetic mode using an Elx800 Microplate Reader (Biotek, USA). The inhibitory capacity of EA (% anticollagenase activity) was determined according to the following equation:

$$\text{Anticollagenase activity } (\%) = \frac{Ac\text{-}As}{Ac} X\ 100 \quad (2)$$

Where A_c is the absorbance of the control and A_s is the absorbance of EA sample.

2.5. Antielastase activity assay

The evaluation of antielastase activity was performed based on the assay described by Thring, Hili and Naughton (2009), using 25 µg of pure EA as well. First, a stock solution of porcine pancreatic elastase at 3.33 mg/mL in sterile ultra-pure Milli-Q water has been prepared. The substrate *N*-Succinyl-*N*-succinyl- (Ala) 3-*p*-nitroanilide was dissolved in 0.2 mM Tris-HCl buffer solution (pH 8.0). EA was incubated with the enzyme for 15 minutes prior to the addition of the substrate. EGCG (250 uM or 0.114 mg/ml) was used as the positive control. Negative controls were performed with ultra-pure Milli-Q water. The absorbance at λ=410 nm was measured immediately after the substrate addition and then continuously during the next 2h using an Elx800 Microplate Reader (Biotek, USA). Inhibition of elastase has been assessed from the release of p-nitroaniline, revealing the substrate proteolysis and subsequent coloring. The antielastase activity (in %) has been calculated using the equation:

$$\text{Antielastase activity } (\%) = \frac{Ac\text{-}As}{Ac} X\ 100 \quad (3)$$

Where Ac is the absorbance of the control group and As is the absorbance of EA sample.

2.6. ^{1}H Nuclear magnetic resonance (NMR)

In order to investigate the interaction between CHI, zein and EA alone or in binary mixtures, we used the following procedure: an amount of each polymer was dissolved together with EA at 1:50 (EA: polymer) in CD_3OD:D_2O 90:10 (v/v), which was homogenized and submitted to analysis using saturation transfer difference (STD) proton nuclear magnetic resonance (NMR) spectroscopy in an Inova 750 spectrometer (Varian, Switzerland) at room temperature. The processing and analysis of the NMR data was performed with the MestreNova® software.

2.7. Statistical analysis

The antimicrobial and antioxidant activities data were submitted to analysis of variance (ANOVA), followed by Tukey post-test for comparison between pairs, assuming a minimum significance level of 5% ($p<0.05$). Results were expressed as mean ± standard deviation. Analyzes were performed using Statistica 7.0 software (StatSoft).

3. Results and Discussion

3.1. Antimicrobial inhibitory activity

The antimicrobial activity of EA, CHI and zein isolated and combined are presented in table 1. The values obtained for MIC and MBC of EA for *S. aureus* were 0.104 and 0.291 mg/ml and for *P. aeruginosa* 0.208 e 0.416 mg/ml, respectively. These values were relatively low demonstrating a high antibacterial activity of this bioactive. When EA was associated to zein, it showed a MIC of 0.104 mg/ml and MBC of 0.166 mg/ml for *P. aeruginosa* showing an significant reduction compared to EA alone. By associating EA with CHI the MIC for *S.aureus* was 0.0625 mg/ml and for *P. aeruginosa* 0.083 mg/ml, which were also significantly lower than the values found with isolated EA. The association of EA simultaneously with zein and CHI showed no significant difference in MIC and MBC for both bacteria when compared to the isolated EA. Hence, it was observed that zein improved the antimicrobial activity of EA against *P. aeruginosa*, whereas CHI improved the inhibitory activity of EA against both bacteria.

EA is a phenolic compound able to decrease the virulence of *P. aeruginosa* at a concentration of 1 mg/ml has decreased its biofilm formation (Sarabhai et al., 2014). Studies by Jayaraman et al. (2010) regarding the activity and interactions of antibiotic to phytochemical combinations against *P. aeruginosa* concluded that 4 mg/ml concentration of EA were able to inhibit the bacterial growth.

The antimicrobial activity of phenolic compounds against *S. aureus* was studied by Rúa et al. (2010), who concluded that EA at 0.200 mg/ml has been able to inhibit the bacterial growth. A similar result was found in the studies of Nayeem and Karvekar (2011), which concluded that EA isolated from the leaves of *Tectona grandis* presented inhibitory activity against *S. aureus* at 0.200 mg/ml. Our findings were superior, as pure EA presented an inhibitory activity at the concentration of 0.104 mg/ml, which decreased down to 0.0625 mg/ml when associated to CHI.

The antibacterial activity of EA was very substantial against gram-positive and gram-negative bacteria. Therefore, the association of zein and CHI to EA under planned conditions may improve its antibacterial performance.

Moreover, in our results it was also possible to demonstrate a relevant antibacterial activity of CHI. The MIC and MBC values of CHI against *S. aureus* were 8.33 and 20.83 mg/ml and for *P. aeruginosa* 10.41 and 41.66 mg/ml, respectively. CHI permeates the membrane of some gram-negative bacteria (*P.aeruginosa, E. coli*), leading to bacteriostatic effect (Helander et al., 2001) and may show also bactericidal activity against some gram-positive bacteria, such as we have demonstrated.

Zein also presented antimicrobial activity against the tested bacteria. The values obtained for MIC and MBC against *S. aureus* were 16.66 and 41.66 mg/ml and for *P. aeruginosa* 10.41 and 16.66 mg/ml, respectively. It was noted that its inhibitory activity occurred against both gram-positive and gram-negative bacteria. This feature lacks knowledge, requiring further investigation.

3.2. Antioxidant activity

The results of the antioxidant activity regarding the DPPH radical inhibition of EA, zein and CHI (Figure 2) show a high antioxidant ability of EA. Additionally, the polymers at the concentrations evaluated also showed a relevant antioxidant activity. The values of IC_{50} obtained for EA, zein and CHI were 0.079, 5.81 and 14.85 mg/ml, respectively. Accordingly, the results obtained herein are in agreement to other authors and reinforce the importance of those biopolymers in the view of their pharmaceutical applications.

IC_{50} of the EA was relatively low, 0.079 mg/ml and when associated to zein presented an IC_{50} of 0.120 mg/ml, despite having a higher IC_{50}, such association may be favorable

When hydrolyzed with alkaline proteases, zein presented 12% antioxidant activity in the concentration of 300 μg/ml (Tang, Zhuang, 2014). In our study, the assayed concentration of zein at 500 μg/ml presented approximately 21% antioxidant activity, confirming its ability to combat free radicals. The antioxidant activity of CHI is related to the degree of deacetylation, the higher this degree the greater this capacity. CHI presented 10% antioxidant activity in the concentration of 3mg/ml (Xing et al., 2005).

The antioxidant ability of EA associated to the polymers is presented in Figure 2. EA in the concentrations of 0.125, 0.075, 0.050 and 0.025 mg/ml associated to zein improved ($p<0.05$) its antioxidant activity, showing that the association of both is very favorable against free radicals enrolled in the oxidative processes. Concentrations between 0.125 and 0.025 mg/ml of EA assayed together with 2.5 mg of zein significantly improved the antioxidant activity of EA at 3% in average (Figure 3).When associated to CHI, there was a significant reduction in the antioxidant activity. Hence, this combination would not be beneficial as it could inhibit its antioxidant activity. The IC_{50} of the EA associated to zein was 0.120 mg/ml, while the association to CHI reached 0.436 mg/ml, showing that the association of EA with zein is more favorable regarding the improvement of this therapeutic target.

3.3. Anticolagenase and antielastase inhibitory activity

The inhibitory effect of EA against collagenase was approximately 66.83%, while its antielastase activity was approximately 48.34%, showing a pronounced inhibitory activity against both enzymes. In the study of Pitchakarn et al. (2013) EA at 25 μg/ml has shown 68% inhibition of

collagenase, a result very similar the obtained in this work. Cho et al. (2011) showed that EA metabolites were able to inhibit the release of elastase by human neutrophils. Thus, the antielastase and anticolagenase activities aforementioned would be another mechanisms to contribute for the skin repairing/preserving and represent a great potential for the therapeutic/cosmetic use of EA.

3.4. Proton NMR studies: characterization of EA-polymers interaction

The chemical interactions between EA and the polymers CHI and zein were evaluated by Proton NMR. The spectra obtained from the STD NMR spectroscopy for EA, CHI and zein are shown in Figures 4 and 5. The ^{1}H of EA has been observed at 7.5ppm. For CHI-EA no interaction has been observed (Figure 4b). In contrast, the spectra of EA assayed together with zein has evidenced clearly the transference toward its aromatic ^{1}H, indicating the presence of drug-polymer chemical interaction. In the study of Sousa et al. (2012) this occurrence has been reported for zein associated to tetracycline, which also presents itself a phenolic structure. For instance, this outcome could explain the observed improvement on the biological properties of EA when associated to zein, comparatively superior to CHI. These findings could contribute to expand its use for new applications, where the improvement of the biological properties attributed to EA could be advantageous.

4. Conclusions

Ellagic acid has demonstrated pronounced bacteriostatic and bactericide activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Zein potentialized its effect against *Pseudomonas aeruginosa*, while CHI increased the inhibitory activity of EA against both strains. Zein and CHI have also demonstrated a reasonable bacterial inhibition against both species when tested alone. EA has exhibited antioxidant activity at all concentrations tested, with an IC_{50} of 0.079mg/ml. Zein solely has exhibited a moderate antioxidant activity and when associated to EA has improved its antioxidant activity. The chemical interaction and pharmacophore conformation towards its availability could explain its enhancement. EA presented pronounced anticollagenase and antielastase activities. Therefore, the association of EA with the polysaccharide CHI and specially the protein zein could benefit its pharmaceutical usage; as such the drug-polymer combinations studied in this paper were able to improve key pharmacological properties attributed to this bioactive. The rational design of pharmaceutical systems based on these combinations would make possible the development of new medicines, where the combinations could reach new therapeutic possibilities.

References

Al-Obaidi, M.M., Al-Bayaty, F.H., Al Batran, R., Hassandarvish, P., Rouhollahi, E. 2014. Protective effect of ellagic acido on healing alveolar boné after tooth extraction in rat – A histological and immunohistochemical study. Arch Oral Biol**.** 52(9), 987-999. doi: 10.1016/j.archoralbio.2014.06.001.

Cardoso, M.J.B., Barbosa, R.C., Ries, A., Fidelis, T., Fook, M.V.L. 2012. Ensaios físico-químicos e mecânicos de membranas de quitosana com cobre, zinco e magnésio. Rev Elet Mat Proc. 1, 1-6.

Cho, J.Y., Lee, T.H., Hwang, T.L., Yang, S.Z., Chen, I.S., Chou, T.H., Sung, P. j., Chen, P. p. 2011. A new ferulic acid ester, a new Ellagic acid derivative, and other constituents from pachycentria formosana: effects on neutrophil pro-inflammatory responses. Chem Biodivers. 8(9), 1709-1716. doi: 10.1002/cbdv.201000228.

CLSI. 2006. Laboratory documents: development and control; approved guideline.5th edition. Clinical and Laboratory Standards Institute, Wayne (PA).

Corradini, E., Curti, P.S., Meniqueti, A.B., Martins, A.F., Rubira, A.F., Muniz, E.C. 2014. Recent advances in food-packing, pharmaceutical and biomedical applications of zein and zein-based materials. Int J Mol Sci. 15(12), 22438-22470. doi: 10.3390/ijms151222438.

Doulabi, A.H., Mirzadeh, H., Imani, M., Samadi, N. 2013. Chitosan/ polyethylene glycol fumarate blend film: physical and antibacterial properties. Carbohydr Polym. 92(1), 48-56. doi: 10.1016/j.carbpol.2012.09.002.

Gopalakrishnan, L., Ramana, L.N., Sethuraman, S., Krishnan, U.M. 2014. Ellagic acid encapsulated chitosan nanoparticles as anti-hemorrhagic agent. Carbohydr Polym. 111(13), 215-221. doi: 10.1016/j.carbpol.2014.03.093.

Helander, I.M., Nurmiaho-Lassila, E.L., Ahvenainen, R., Rhoades, J., Roller, S. 2001. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microb. 71, 235-244. doi: 10.1016/S0168-1605(01)00609-2.

Jayaraman, P., Sakharkar, M.K., Lim, C.S., Tang, T.H., Sakharkar, K.R. 2010. Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa in vitro.* Int J Biol Sci. 6(6), 556-568. doi: 10.7150/ijbs.6.556.

Nayeem, N., Karvekar, M. 2011. Antimicrobial and anti-oxidant properties of the isolated compounds from the methanolic extract from the leaves of *Tectona grandis*. *J* Basic Clin Pharm. 2(4), 163-165.

NCCLS. 2006. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 7th edition. Clinical and Laboratory Standards Institute, Wayne (PA).

Pitchakarn, P., Chewonarin, T., Ogawa, K., Suzuki, S., Asamoto, M., Takahashi, S., Shirai, t., Limtrakul, P. 2013.. Ellagic acid inhibits migration and invasion by prostate cancer cell lines. Asian Pac J Cancer Prev. 14(5), 2859-2863. doi: 10.7314/APJCP.2013.14.5.2859.

Rúa, J., Fernández-Álvarez, L., Gutiérrez-Larraínzar, M., Del Valle, P., Arriaga, D., García-Armesto, R. 2010. Screening of phenolic antioxidants for their inhibitory activity against foodborne *Staphylococcus aureus* strains. Foodborne Pathog Dis. 7(6), 695-705. doi: 10.1089/fpd.2009.0440.

Sarabhai, S., Harjai, K., Sharma, P., Capalash, N. 2015. Ellagic acid derivates from *Terminalia chebula Retz.* increase the susceptibility of Pseudomonas aeruginosa to stress by inhibiting polyphosphate kinase. J Appl Microbiol. 118(4), 817-825. doi: 10.1111/jam.12733.

Shahram, E., Sadraie, S.H., Kaka, G., Khoshmohabat, H., Hosseinalipour, M., Panahi, F., Nimi-Jamal, M.R. 2013. Evaluation of chitosan-gelatin films for use as postoperative adhesion barrier in rat cecum model. Int J Surg. 11(10), 1097-1102. doi: 10.1016/j.ijsu.2013.09.012.

Sousa, C.M.M., Silva, H.R., Vieira-Jr, G.M., Ayres, M.C.C., Costa, C.L.S., Araújo, D.S., Cavalcante, L.C.D., Barros, E.D.S., Araújo, P.B.M., Brandão, M.S., Chaves, M.H. 2007. Fenóis totais e atividade antioxidante de cinco plantas medicinais. Quim Nova. 30(2), 351-355. doi: 10.1590/S0100-40422007000200021

Sousa, F.F.O., Blanco-Méndez, J., Pérez-Estévez, A., Seone-Prado, R., Luzardo-Álvarez, A. 2012a. Effect of zein on biodegradable inserts for the delivery of tetracycline within periodontal pockets. J Biomater Appl. 27(2), 187-200. doi: 10.1177/0885328211398968.

Sousa, F.F.O., Luzardo-Álvarez, A., Blanco-Méndez, J., Martín-Pastor, M. 2012b. NMR techniques in drug delivery: application to zein protein complexes. Int J Pharm. 439(1-2), 41-48. doi: 10.1016/j.ijpharm.2012.09.046.

Tang, N., Zhuang, H. 2014. Evaluation of antioxidante activities of zein protein fractions. J Food Sci. 79(11), 2174-2184. doi: 10.1111/1750-3841.12686

Thring, T.S., Hili, P., Naughton, D.P. 2009. Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. BMC complement Altern Med. 4, 9-27. doi: 10.1186/1472-6882-9-27

Xing, R., Liu, S., Guo, Z., Yu, H., Wang, P., Li, C., Li, Z., Li, P. 2005. Relevance of molecular weight of chitosan and its derivatives and their antioxidante activities *in vitro*. Bioorg Med Chem. 13, 1573-1377. doi: 10.1016/j.bmc.2004.12.022.

Table 1. Antimicrobial activity of EA and the polymers CHI and zein (mean ± SD).

Tested Samples	MIC (mg/ml)		MBC (mg/ml)	
	S. aureus	***P. aeruginosa***	***S. aureus***	***P. aeruginosa***
EA	0.104 (±0.036) [a]	0.208 (±0.072) [a]	0.291 (±0.190)[a]	0.416 (±0.144) [a]
ZEIN	16.66 (±7.21)	10.41 (±3.6)	41.66 (±14.43)	16.66 (±7.27)
CHI	8.33 (±3.60)	10.41 (±3.6)	20.83 (±7.21)	33.33 (±14.43)
EA + zein	0.125[a]	0.104 (±0.030) [bc]	0.375(0.176)[a]	0.166 (±0.072)[b]
EA + CHI	0.0625[b]	0.083 (±0.030)[c]	0.250[a]	0.375 (±0.176)[a]
EA+ zein+ CHI	0.104 (±0.036) [a]	0.166 (±0.049) [ab]	0.250[a]	0.333 (±0.144) [a]

Same letter in the same column indicates that there is no significant difference between the mean values (Tukey test, $p < 0.05$). Only EA values were considered in the combinations with the polymers. The polymers initial concentration used was 50 mg/ml.

Figure 1. Chemical structure of Ellagic acid.

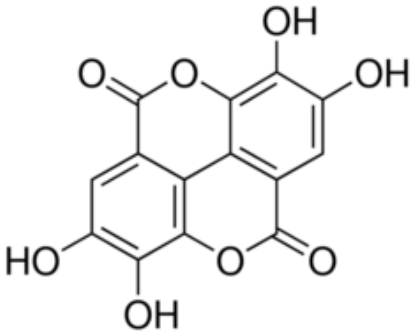
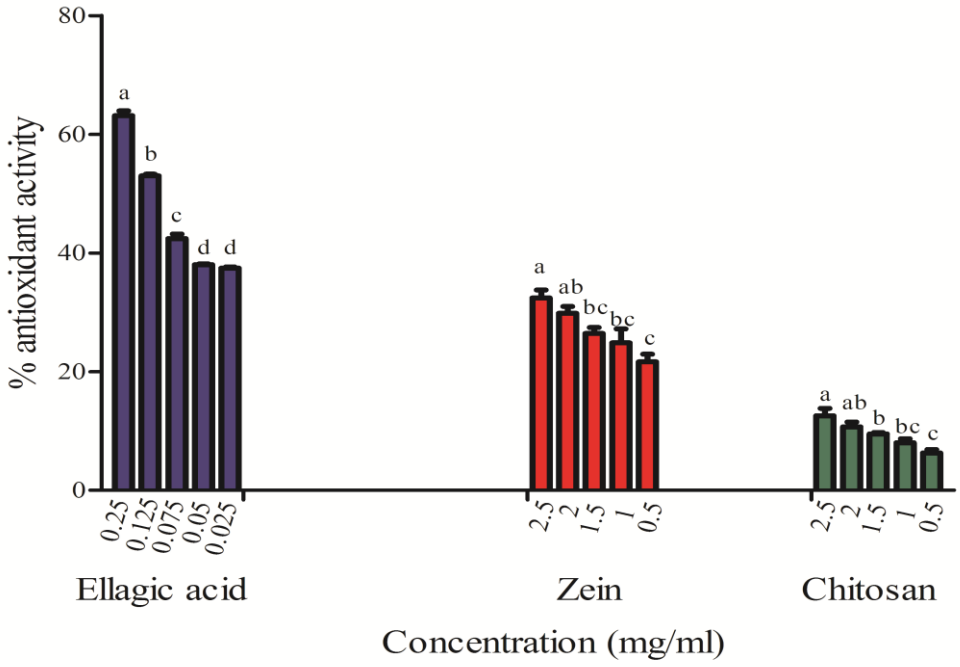
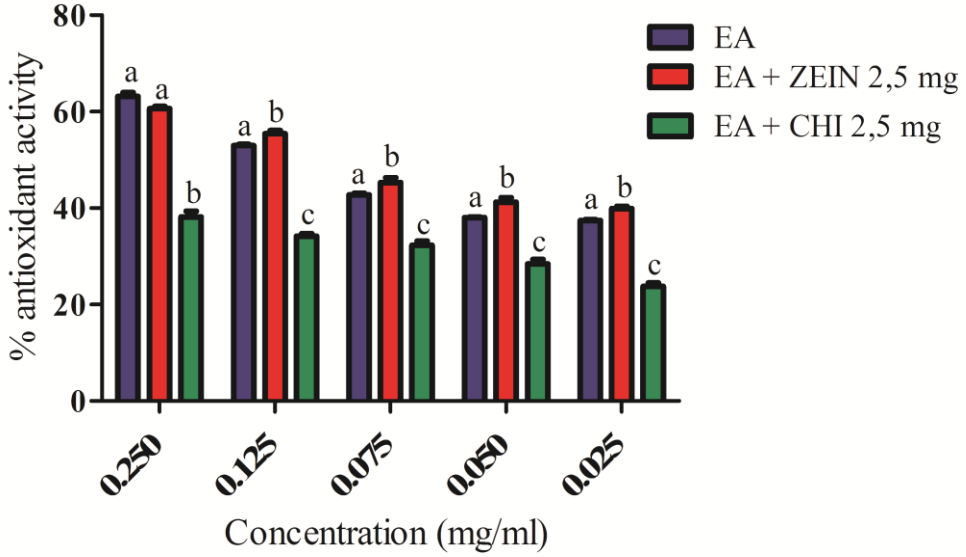


Figure 2. Antioxidant responses of ellagic acid, chitosan and zein



Same letter in the same group indicates that there is no significant difference between the mean values (Tukey test, $p < 0.05$).

Figure 3. Antioxidant activity of ellagic acid associated to chitosan and zein.



Same letter in the same group indicates that there is no significant difference between the mean values (Tukey test, $p < 0.05$).

Figure 4. H^+ NMR spectra of CHI and EA. a) 1H spectrum of the mixture CHI-EA, b) STDoff-on spectrum of the mixture CHI-EA with the on-saturation applied over a signal of zein at 2.0 ppm (indicated with a ray symbol). c) 1H spectrum of the pure EA sample. and d) 1H spectrum of pure CHI sample. The assignment is provided in spectrum a), (*) impurities, (m) methanol, (e) ethanol.

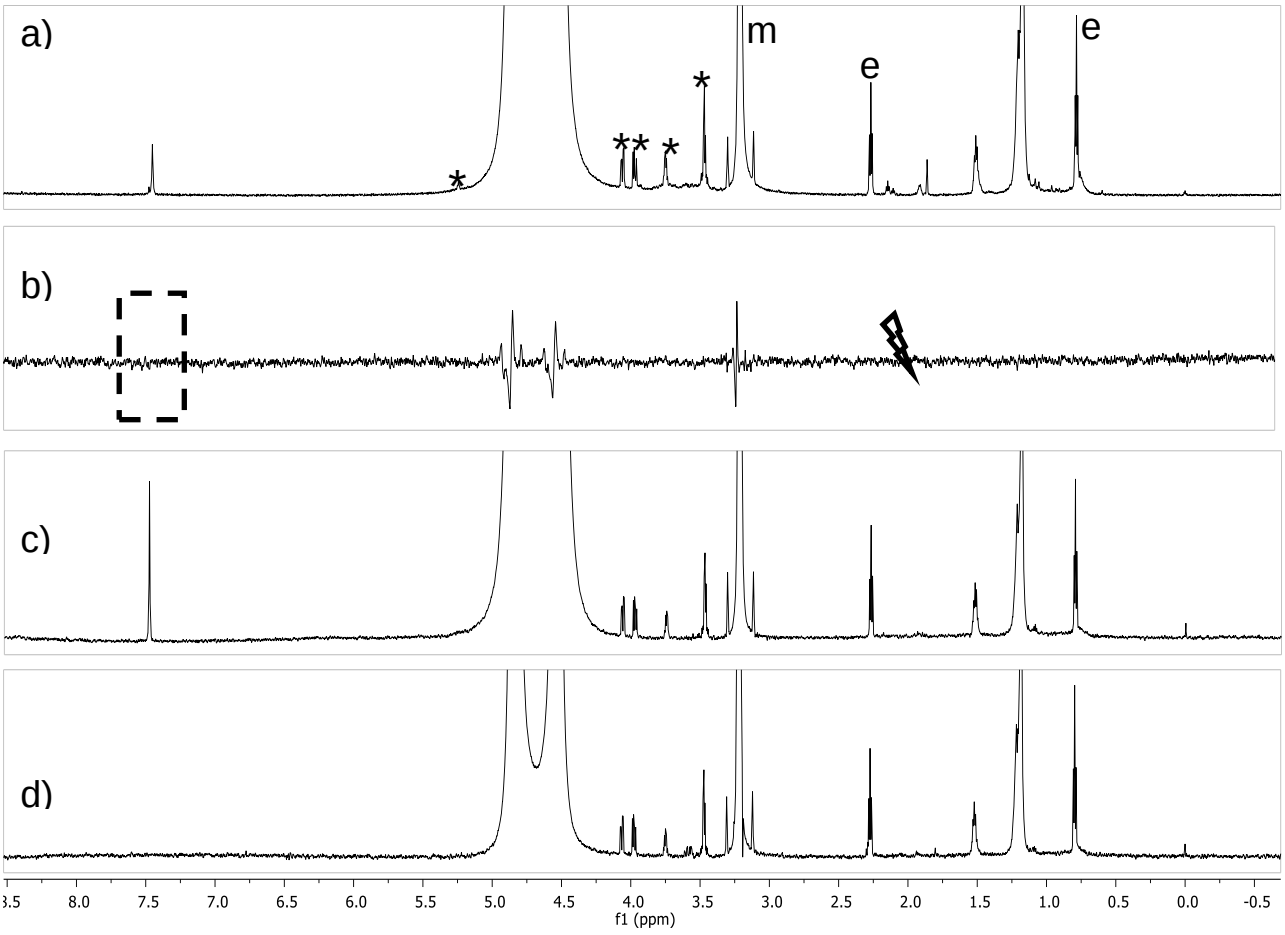
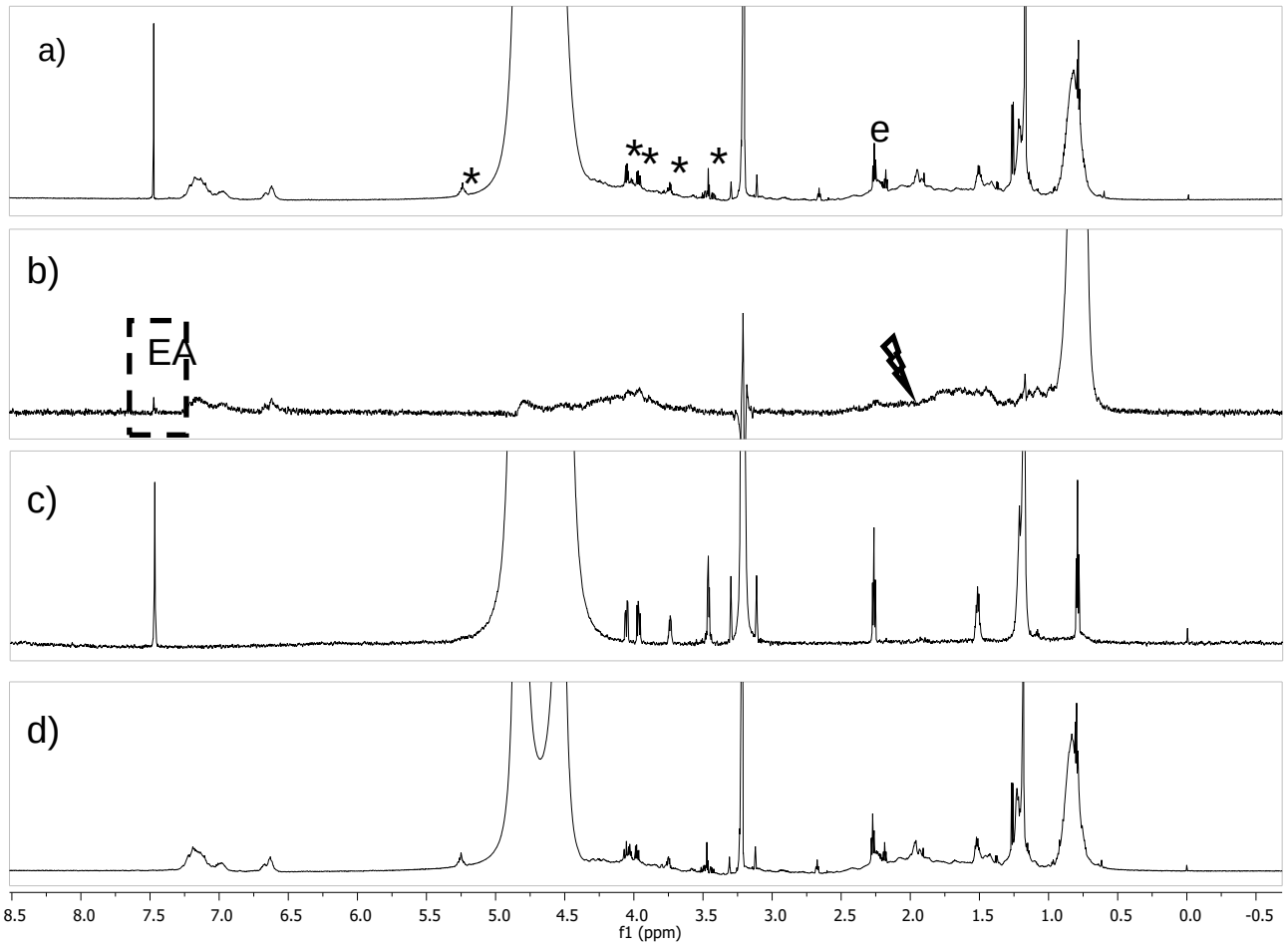


Figure 5. H^+ NMR spectra of zein and EA. a) 1H spectrum of the mixture zein-EA, b) STD^{off-on} spectrum of the mixture zein-EA with the on-saturation applied over a signal of zein at 2.0 ppm (indicated with a ray symbol). c) 1H spectrum of the pure EA sample. and d) 1H spectrum of pure zein sample. The assignment is provided in spectrum a), (*) impurities, (m) methanol, (e) ethanol.



5 CAPÍTULO 3

DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN/ZEIN FILMS FOR CONTROLLED RELEASE OF ELLAGIC ACID

Artigo a ser submetido para publicação ao Journal of Biomedical Materials Research Part A:*

**A formatação deste capítulo segue as normas do Journal of Biomedical Materials Research Part A.*

Development and characterization of chitosan/zein films for controlled release of ellagic acid

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ABSTRACT

Chitosan/zein films have been developed for controlled release of ellagic acid. A central composite design with $\alpha = \sqrt{2}$ was used to evaluate the effect of the polymer concentrations on the responses: film thickness, water uptake, solubility, contact angle, *in vitro* release and antimicrobial activity. The mechanical properties have been also accessed. In addition, the release profiles have been studied by fitting to mathematical models. The morphology and possible interactions between the film components were studied by SEM and FTIR analysis. The analysis of variance of the experimental data shows that the characteristics can be well predicted with quadratic models. The responses depended on the composition of the film, with both polymers exerting a significant influence on the thickness and the contact angle ($p<0.05$). Zein presented a significant influence on the release of ellagic acid. Moreover, the obtained films presented suitable thickness, high sorption, low solubility, slow and controlled release and inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In view of these results, the polymeric films obtained could be further investigated as a treatment option to rehabilitate the skin/connective tissues or any other conditions whose would take advantage from the biopharmaceutical properties of ellagic acid.

KEY-WORDS: Polymeric films. Ellagic acid. Chitosan. Zein. Antimicrobial activity. *In vitro* release

INTRODUCTION

Polymers are valuable materials used in different forms in pharmaceutical purposes. One of these applications is films, which can be impregnated with bioactives with diverse pharmacological applications. Among the most used polymers nowadays is chitosan (CHI), a natural polysaccharide found in crustacean shells that presents a great potential in cutaneous treatments[1], due to its biocompatibility, biodegradability and bioadhesiveness[2] associated to its antimicrobial potential[3]. This polymer has already been studied in association with different substances such as zein[4] and ellagic acid[5-6] , whose are assayed together in the current paper.

Zein is a natural protein found in the maize (*Zea mays*) endosperm[7] in the amorphous form, resulting in a plasticizing viscoelastic appearance[8]. Recently, it has been used in the composition of some drug delivery systems, such as nano/microparticles[9-13].

Ellagic acid (EA) is an ellagitannin found in fruits like pomegranates. Diverse biological activities are attributed to it, such as: antimicrobial[13-15], antioxidant[16], healing[17] and antihemorrhagic[18].

Considering the biopharmaceutical characteristics of EA and the filmogenic, biodegradable and biocompatible characteristics of CHI and zein, they had been employed to design CHI/zein

films for controlled release EA, which could serve as a platform for tissue rehabilitation, with regards to the biopharmaceutical characteristics of EA.

MATERIALS AND METHODS

Materials

Chitosan (deacetylation degree = 85%, molecular weight = $1{,}9X10^5$ Da), zein, ellagic acid (purity ≥95%) were purchased from Sigma-Aldrich (USA). All the other reagents were pure grade and used according to its manufacturer specifications.

Experimental design and statistical analysis

In this study, the influence of the polymer concentrations on the properties - thickness, water uptake, solubility, contact angle, *in vitro* release of EA and antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* - of the films were evaluated using the central composite design 2^2 with $\alpha = \sqrt{2}$. A total of 13 films were prepared, using 4 factorial points (levels -1 and +1), 4 axial points (level ± α) and 5 central replicates (0,0) (Table I). The statistical significance of each individual coefficient was determined by evaluating the *p-value* with a 95% confidence level obtained from the analysis of variance (ANOVA). The experimental design and the statistical analysis were performed using the software Statistica7.0 (StatSoft).

Films preparation

The films were prepared using the casting method. Briefly, CHI solutions were prepared from its dissolution in 3%v/v acetic acid and glycerol 6.25%v/v. Separately, zein was dissolved in 70% ethanol, where 1.2%w/v EA has been added. After that, the solutions were mixed and stirred until its complete homogenization, being transferred into Petri dishes for solvent evaporation at 25°C. After total evaporation, the membranes were neutralized with NaOH 2N solution and washed with distilled water and dried again. After that, they were stored in sealed plastic bags protected from light and humidity until its further usage.

Scanning electronic microscopy (SEM) analysis

The surface morphology of the films was examined by scanning electron microscope TM3030 Plus (Hitachi, Brazil). The images were recorded in the electric scattering mode 15 kV and recorded in 1.0K magnification.

Thickness

The film thickness was measured using a digital caliper (ZAAS Precision, Brazil). Six different regions of each film have been measured, and the mean has been considered as the film thickness[19].

Water uptake and Solubility

The water uptake ability and solubility of the films have been evaluated as follows: five samples of 1 cm^2 of each film were oven dried at 40°C for 48 hours and weighed (W_1). After that, the samples were immersed in 15 ml distilled water during 4 days. After this period, the samples were collected, the excess of water was carefully removed with absorbent paper, and weighed again (W_2) to determine its water uptake capacity, according to the equation:

$$\text{Water Uptake capacity}(\%) = \frac{(W1-W2)}{W1} \text{ X } 100 \quad (1)$$

Where W_1 is the initial mass and W_2 is the wet film mass.

The samples were again placed in an oven at 70ºC until reaching a constant weight (after 3 successive measurements) (W_3) to determine the amount of material remaining in the solution. Thus the solubility was calculated according to the equation:

$$Solubility(\%) = \frac{(W1-W2)}{W1} X\ 100 \quad (2)$$

Where W_1 is the initial and W_3 is the final mass.

Contact angle

The contact angle was evaluated according to the methodology used by Sousa et al.[8] where a drop of 25 µl of distilled water was placed on the surface of each film at 25±2°C, 53±2% relative humidity, and the images were recorded with a digital camera in macro mode (Sony® model

W570). The contact angle was determined using the Meazure ™ 2.0 (C Thing Software, USA) image platform. The tests were performed in triplicate.

Fourier transform-infrared spectroscopy (FTIR)

The infrared spectra of the films were recorded using a FTIR-ATR spectrophotometer IRAffinity-1 (Shimadzu, Brazil) in the range of 400 to 4000 cm^{-1}, with a resolution of 4 cm^{-1} and a minimum of 12 scans. The films were placed directly in the sample support without any previous preparation and scanned individually.

***In vitro* release assay**

The release test of the films was performed using a vertical Franz diffusion cells system (Spell Glass 3, Brazil) in a shaker SL-222(Solab, Brazil) at 37±1ºC over a 48h period. A sample of 2.25 cm^2 of each film, previously hydrated with distilled water, was placed between the donor and receiver compartments of the vertical cell. In the receiver compartment, 6.0ml of saline phosphate-buffer (PBS, pH 7.4) was placed, while no fluid was placed on the donor compartment. The system was stirred constantly during the test. At predetermined time intervals (1, 2, 3, 4, 7, 11, 24 and 48 hours), 1.0 ml aliquots were collected and replaced with an equivalent amount of fresh PBS. The amount of EA released into the receptor solution was determined by UV-VIS spectroscopy (Biospectro SP-22, Brazil) at a wavelength of λ=345 nm, using the following calibration curve: y = 4.28858x - 0125, R^2=0.999. The release behavior was also characterized by evaluating the adjustment of the releasing curves to zero order, Higuchi[20], Korsmeyer-Peppas[21] and Peppas-salin[22] models. The software Origin® 8.0 was used for that purpose.

Antimicrobial activity evaluation

The antimicrobial activity was assessed using Kim et al[6] methodology with some modifications. The bacteria used in this study were *Staphylococcus aureus* (ATCC 6538P) and *Pseudomonas aeruginosa* (ATCC 9027), due to its major implication in critical wound infection[14-15]. Both were grown in sterile BHI broth at 37±1°C for 18h. After culturing, the concentration was adjusted to $1.5x10^8$ colonies forming units/mL (CFU/mL) in sterile BHI broth (solution I) and the

bacterial inoculum was prepared at 1:100 dilution of solution I. In a sterile plastic microtube, 1.35 ml of sterile BHI broth, 150 μl of the bacterial inoculum and a square sample of 0.6x0.6 cm of each film were incubated at 37±1°C for 24 h. Microtubes containing sterile BHI broth were used as controls. After the incubation period, a serial dilution up to 10^{-5} of the broth was performed. From each dilution, aliquots of 10 μl were plated on Mueller-Hinton agar and incubated at 37±1°C for 48 h. After the incubation time, the CFU were determined and corrected to each dilution factor in order to determine the antibacterial activity related to each film. Samples of each film were also wet sterilized at 121ºC for 15 min in order to determine the effect of the sterilization on their performance. The test was performed in triplicate.

Mechanical properties

The mechanical properties were evaluated for the films that presented the best responses in terms of thickness (*adequate*), water uptake (*high*), solubility *(low)*, hydrophilia, surface (*smooth and dense*) and high *in vitro* release of EA and inhibitory activity against *S.aureus* and *P.aeruginosa*. Films without EA and a film made only of CHI (without zein or EA) have also been assayed as controls. The test was performed based on standardized methodology ASTM D882-02[23]. The films were cut into rectangular strips (75mmX10mm). The tensile strength (TS) and the percentage of elongation at break (E%) were measured using a universal testing machine DL2000 (Emic, Brazil) in the tensile mode, using a load cell of 500N and deformation rate of 10 mm/min. The E% was calculated from the difference between the initial sample length and the extended length at the moment of breakage[6], where the TS has been determined.

RESULTS AND DISCUSSION

Films morphology

All the films looked slightly yellow and were flexible and easy to handle. Visually the EA was mostly dispersed uniformly within the film. The thickness, water uptake, solubility and contact angle of the films are shown in Table II.

The thickness of the polymer films varied according to the concentration of CHI and zein, and there was a significant difference ($p<0.05$) between F11 and F10 films. This may have occurred due to the difference in CHI concentration because CHI is more dense than zein[4] directly influencing the formation of thicker films[24].

The films SEM images are shown in figure 1. It can be observed that there was a relevant difference among the films, which may have occurred mainly due to the quali and quantitative differences in the polymers used in each formulation. The films F1, F2 and F10 presented a dense, rough surface and apparently there was not a total dispersion of EA within the films structure. F3, F4 and F11 films presented a fairly porous surface. The films F5-F9, F12 and F13 presented a more uniform, denser and smoother surface than the other films. Studies involving the development of CHI films have shown a dense and smooth or porous surface[6,25], what will depend on the film composition and the preparation method enrolled. Zein in certain conditions could also result in aggregation itself[9].

Water uptake, Solubility and hydrophilicity of the films

All the obtained films presented a sorption level over 100%. The F3 film presented a higher sorption than the films F2, F11 and F12. The differences in the polymer concentrations may explain this finding, once the higher percentage of CHI in F3 would contribute for its hydrophilicity[19]. Besides, the polymeric structure of the film may have lead to this difference, as F3 films presented a dense appearance with some pores whereas F11 presented a highly porous appearance. For instance, F2 and F12 presented a denser appearance (figure 1). CHI presents a hydrophilic nature, in which the hydroxyl and amino groups of its structure have the ability to interact with water molecules[19], as such; zein when mixed to CHI could increase this barrier[4].

Some films presented the solubility level inferior to 10% (F1, F2, F3, F4, F5-F9, F13), in contrast to the remaining films (F10, F11, F12), which presented it higher. This fact might be attributed to the instability in the films formation related to the polymer conjugation, once the films with the lowest (F10) and the highest (F11) concentration of CHI and with the lowest concentration

of zein (F12) showed significantly higher solubility than the other films. The relatively low solubility presented by these films are strictly correlated to the characteristics of CHI that possess little water solubility[26] and zein that produces films reasonably resistant to water due to its notorious hydrophobia[27].

As for the hydrophobicity, the films exhibited a variation in contact angle from 60.52 to 99.9°, which points its majorly hydrophilic surfaces[19]. The films F2, F10 and F13 presented a contact angle over 98.5°, being significantly different from others. This different is attributed to the fact that these compositions contain a higher proportion of zein and as a result a greater hydrophobic nature[27]. Moreover, the films containing higher proportions of CHI presents lower contact angles, as result of the hydrophilic nature of this polymer[28].

For instance, most films presented favorable characteristics for use in the treatment of wounds or other skin/connective tissue disorders: suitable thickness, high water sorption, low solubility and wettability according to the contact angle. Such characteristics are essential to maintain a humid environment which promotes the healing and decrease the exudates present in wounds[6].

Fourier transform-infrared spectroscopy (FTIR)

The individual IR spectra of CHI, zein and EA are shown in figure 2. The spectrum obtained for CHI and zein shows its most characteristic bands. Both exhibit axial stretching of OH, superimposed on the N-H stretch band at 3379 and 3290 cm^{-1}. The C-H stretch appears at 2870 cm^{-1} band on the CHI and 2966 cm^{-1} on the zein. The C = O axial deformation of the primary amide is at 1645 cm^{-1} band on the CHI and 1654 cm^{-1} on the zein. The axial deformation of C-N is at band 1305 cm^{-1} in CHI and 1238 cm^{-1} in zein. Bands 1356 and 1537 cm^{-1} are attributed to the N-H axial deformation in CHI and zein, respectively[29-30]. The EA spectra shows a band at 3149 cm^{-1} corresponding to the axial stretch of OH. The band at 1656 cm^{-1} is associated to the aromatic C=C stretch. The 1600 cm^{-1} band is attributed to the C-C stretch. The band 1710 cm^{-1} corresponds to the C = O stretch. The band 1157 cm^{-1} is associated to the C-O-C. The band 1342 cm^{-1} corresponds to

the hydrogen bonds (HO...H). The band 758 cm^{-1} indicate the presence of the phenyl ring and the band at 723 cm^{-1} is associated to the C-H angular deformation[31].

The spectra of the films are shown in figure 3, where it is noticeable a great similarity. All samples showed axial stretching of OH, superimposed on the NH stretching between the 3370 and 3390 cm^{-1} bands, which may indicate an interaction of the -OH group of EA with the -NH groups of CHI and/or zein through hydrogen bonds, forming of a strong network struture[19]. The C=O stretch of amide I (CHI and zein) was displaced between 1662 and 1670 cm^{-1} bands in all films as a result of the presence of polyols (EA) forming hydrogen bonds with the amine groups of CHI and/or zein[31]. The angular deformation of the N-H bond of amide II in zein spectra is noted between the bands 1531 and 1545 cm^{-1}. The phenyl ring of EA was displaced in the films from 800 to 815 cm^{-1}, what could indicate the presence of aromatic condensation within the aromatic aminoacids present in zein. The C-H angular deformation of EA appears between the bands 721 and 725 cm^{-1}. Our results are similar to those reported by Gopalakrishnan et al.[18] and Arulmozhi, Pandian and Mirunalini[31]. Those authors studied EA encapsulated in CHI nanoparticles and concluded that there is a balance between the associative and repulsive forces between EA and CHI chains, predominantly associated to hydrogen bonds evidenced by the displacement of OH stretch and NH_2, as aforementioned. Zein could also play an important role in the entrapment of EA within the films, notably by the aromatic stacking above mentioned and described mechanistically in the studies of Sousa et al[10].

Ellagic acid release profiles from the films

The EA release curves from the polymeric films developed are shown in figure 4. The EA release from the films was slow, increasing reasonably over time. This effect can be attributed to the time required for film hydration[6]. In our study, the concentration of EA in the films was approximately 1.2% and the release observed was ranged among the films from 0.291 μg to 0.374 μg after 48 h. This release is related to the lower contact of the solution with the film, because the adjustment to the mathematical Peppas-Sahlin model shows that the release of EA from the film is

influenced by the relaxation of the polymer chain. This slow and sustained release of EA from the film was already shown in another study, where films containing a 1% EA were immersed in 6.0 ml of PBS and released approximately 21.54 μg after 48h[6], such releasing can be related to prolonged contact of the solution with the film during the immerse.

The overall EA release from the films presented a sustained behavior, though the kinetic varied according to the polymer concentration of each preparation. The films that released more EA after 48h were F13, F2, F10 which reached 6.4, 4.3 and 3.8%, respectively. These films had a higher proportion of zein to CHI in their formulations. Thus, despite its hydrophobicity, zein has increased the release levels within the films, such as it has been observed in other deliver systems[7-10].

The fitting models (zero order, Higuchi, Korsmeyer-Peppas and Peppas-Sahlin) adjusted to the release curves for the films are presented in table III. The EA release from the F1, F2, F3, F4, F11, F13 films followed Korsmeyer-Peppas, while F5-F9, F10 and F12 followed zero order model, according to the adjusts observed.

The Korsmeyer-Peppas model allows characterizing the release mechanism through the value of n[20-21]. Most films presented n> 1.0 characterizing a super transport case II release, which occurs due to combination of Fickian diffusion, film swelling, relaxation and erosion of the polymer chain mechanisms.

In the Peppas-Sahlin model the constants K_1 and K_2 represent, respectively, the influence of diffusion and polymer relaxation. The higher value of K_2 means the greater influence of the polymer relaxation on the release than the diffusion itself[22]. This behavior was noticed among all films, where K_2 was nominally higher than K_1, which suggests that the release of EA has been more influenced by polymeric relaxation than diffusion mechanism.

Antimicrobial inhibitory activity

The antimicrobial activity against *S.aureus* and *P.aeruginosa* was evaluated by means of bacterial viability reduction. The results were expressed in terms of CFU/mL. Figures 5 and 6 show

the antimicrobial activity of the films against *S.aureus* and *P.aeruginosa*, respectively. Most films have reduced expressively the bacterial viability in both bacteria strains. Nonetheless, the F2E and F11E sterilized films had no significant difference in the reduction of *S. aureus* viability when compared to the control group. Nonetheless, F11 and F11E films did not present a significant reduction on *P.aeruginosa* viability in comparison to the control group. The F10, F10E, F2, F3 and F13 films presented a great inhibitory activity against *S. aureus*. Most films present a great inhibitory activity against *P.aeruginosa*, when compared to the control group, except for F3, F3E, F4, F11 and F11E films, whose seems to have a limited effect.

When comparing the effect of the sterilized and non-sterilized films, it was observed that the sterile F2, F3, F5-F9 and F11 films presented significantly lower antimicrobial activity against *S. aureus* compared to non-sterile pairs. Regarding the antimicrobial activity against *P.aeruginosa*, the samples of sterile films presented no significant difference compared to the non-sterile ones. Overall, the film F10 and F10E presented the most pronounced activity among all films obtained, showing that its composition, based on a higher proportion of zein would benefit the antibacterial activity of EA against gram positive and negative bacteria.

The antimicrobial activity of the polymeric films was in good agreement with the results from the release assay. EA has shown the ability to decrease the virulence of *P.aeruginosa*[14] and is able to inhibit *S. aureus* growth[15]. However, this reduction in bacterial viability may have occurred as a result of the combination of the polymers with EA, once CHI has already shown bacteriostatic effect against gram-negative and may have a bactericidal effect against some gram-positive bacteria[3]. Therefore, minimization of bacterial viability may be a result from the synergistic effect of EA with the polymers used.

Mechanical properties

The mechanical properties were evaluated in the films F10 and F13 (table IV), as they presented the best results regarding the physical and biological parameters evaluated above, and thus they would represent the best candidates for further investigations. Briefly, they presented

adequate thickness, high water uptake, relatively low solubility, hydrophilic surface, smooth and dense surface, slow and controlled release superior to the other films and satisfactory inhibitory activity against *S.aureus* and *P.aeruginosa*. They were compared to CHI 2% films, prepared on the same manner as the other films used in this study, except for the absence of zein.

There was no significant difference between F10 and F13 films regarding the tensile strength. However, the CHI film showed a significantly greater difference from F10 and F13 films, once CHI alone is more elastic than zein[4, 9] and thus would produce a more rigid structure related to the microcrystalline domains in its inner structure[26]. As a result, a film based on CHI alone would make it difficult to use in skin or soft tissue lesions[24]. Regarding the elongation, F10 did not present a significant difference for the F13, both containing EA. The incorporation of EA has not influenced the elongation of F10 and F13 films individually compared to their pairs without EA. Our results do not agree to those of Escamilla-Garcia et al.[4] who studied CHI/zein films and concluded that there was no significant difference between CHI films and CHI/zein in terms of tensile strength and elongation. Both F10 and F13 films showed adequate tensile strength for wound coverages, because studies on the tensile strength of human skin show values ranging from 21.6 MPa to 28 MPa and an elongation between 25.45 and 54%, which vary according to the person's age[32-33]. Thus, the tensile strength of the films F10 and F13 were nearest to that of the human skin than the CHI film. For instance, the elongation of F13 was superior to F10, meaning greater flexibility[23], which facilitates skin/tissue adaptation. Therefore, both would be suitable for practical usage.

Correlation between films composition and physical/biological properties

The properties of the polymer films have been influenced directly by the concentration of each polymer used. The effect of the concentration of CHI (X_1) and zein (X_2) on the films properties, denoted by the dependent responses are shown in table 2. These results were adjusted in polynomial models and submitted to analysis of variance (ANOVA) to state the statistical significance of the model.

$$\text{Thickness}=190.43-214.05X_1-185.99X_2+0.00X_1X_2-540.62X_1^2+331.66X_2^2 \quad (3)$$

$$\text{Solubility}=16.72-50.84X_1+0.31X_2+7.07X_1X_2+63{,}85X_1^2-6{,}24X_2^2 \quad (4)$$

$$\text{Water uptake}=43.25+402.3X_1+231.69X_2-309.71X_1X_2-323.84X_1^2-78.15X_2^2 \quad (5)$$

$$\text{Contact angle}=118.28-243.34X_1-67.34X_2-100.43X_1X_2+307.42X_1^2+209.02X_2^2 \quad (6)$$

$$\text{Release of EA}=1.09+4.69X_1+2.93X_2-5.8X_1X_2-6.81X_1^2+6.48X_2^2 \quad (7)$$

$$A.A\ S.aureus = 199581.6 + 3077937.4X_1 + 1122249.09X_2 + 5016772.48X_1X_2 - 4880447X_1^2 + 4337093.69X_2^2 \quad (8)$$

$$A.A\ P.aerug. = 58497953.3 - 154316349.2X_1 + 1122249.09X_2 + 5016772.48X_1X_2 + 203570654.33X_1^2 + 62631262.26X_2^2 \quad (9)$$

The polynomial equations above quantify the effects of factors X_1 and X_2 and their interaction on dependent variables. The signal and magnitude of the effects indicate the relative impact of each factor on the response. A negative sign expresses an antagonistic effect and a positive sign expresses synergism[34].

The effect of each factor and its respective *p-value* are displayed in table V, considered significant with *p-value* <0.05. It can be observed that the thickness is influenced by the synergic linear contribution of X_1 and X_2 and quadratic contribution of X_1. The contact angle of the films is negatively influenced by the linear contribution of X_1, synergistically by the linear contribuition of X_2 and by the quadratic contribution of X_1 and X_2 synergistically. The EA release has been only influenced by the synergistic linear contribution of X_2. Solubility, water uptake and antimicrobial activity against *S. aureus* and *P. aeruginosa* were not significantly influenced ($p<0.05$) by none of the independent variables X_1 or X_2.

The dependent variables were adjusted to the polynomial model resulting in the following coefficient of determination (R^2): thickness $R^2=0.9161$, solubility $R^2=0.3830$, water uptake $R^2=0.1408$, contact angle $R^2=0.9624$, EA cumulative release $R^2=0.7627$, antimicrobial activity

against *S.aureus* and *P.aeruginosa* R^2=0.4995 and 0.5197, respectively. It was observed that the variables thickness and contact angle presenting adjustments to the model over 0.9, presented good correlation between the experimental and predicted values[34]. Conversely, the other variables presented a reduced adjustment to the model, evidenced by the R^2 values above.

Although most responses showed a limited fitting to the proposed model, it was observed that the CHI/zein films containing EA presented favorable physical and biological characteristics for its use as a promising treatment film for wounds and/or other biomedical conditions where the pharmacological properties of EA may be beneficial. However, the film F13 showed more favorable characteristics for future research, it had adequate thickness, high sorption, low solubility, hydrophilic surface, smooth and dense, slow and controlled release superior, inhibitory capacity against the bacteria tested, tensile strength and adequate elongation , characteristics that would favor the rehabilitation of the continuity of the skin or any other infected or potentially infectious tissues.

REFERENCES

1. Shahram E, Sadraie SH, Kaka G, Khoshmohabat H, Hosseinalipour M, Panahi F, *et al*. Evaluation of chitosan-gelatin films for use as postoperative adhesion barrier in rat cecum model. Int J Surg. 2013; 11(10): 1097-1102.

2. Doulabi AH, Mirzadeh H, Imani M, Samadi N. Chitosan/ polyethylene glycol fumarate blend film: physical and antibacterial properties. Carbohydr Polym. 2013; 92(1): 48-56.

3. Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microb. 2001; 71: 235-244.

4. Escamilla-García M, Calderón-Domínguez G, Chanona-Pérez JJ, Farrera-Rebollo RR, Andraca-Adame JÁ, Arzate-Vásquez I, et al. Physical and structural characterisation of zein and chitosan edible films using nanotechnology tools. Int J Biol Macromol. 2013; 61: 196-203.

5. Kim S, Gaber MW, Zawaski JA, Zhang F, Richardson M, Zhang XA, et al. The inhibition of glioma growth in vitro and in vivo by a chitosan/Ellagic acid composite biomaterial. Biomaterials. 2009; 30(27): 4743-4751.

6. Kim JO, Noh JK, Thapa RK, Hasan N, Choi M, Kim JH, et al. Nitric oxide-releasing chitosan film for enhanced antibacterial and *in vivo* wound-healing efficacy. Int J Biol Macromol. 2015; 79: 217-225

7. Corradini E, Curti PS, Meniqueti AB, Martins AF, Rubira AF, Muniz EC. Recent advances in food-packing, pharmaceutical and biomedical applications of zein and zein-based materials. Int J Mol Sci. 2014; 15(12):22438-22470.

8. Wang HJ, Ren QS, Wang JY. Applications and degradation of proteins used as tissue engineering materials. Materials. 2009; 2: 613-635.

9. Sousa FFO, Blanco-Méndez J, Pérez-Estévez A, Seoane-Prado R, Luzardo-Álvarez A. Effect of zein on biodegradable inserts for the delivery of tetracycline within periodontal pockets. J Biomater Appl. 2012; 27(2): 187-200.

10. Sousa FFO, Luzardo-Álvarez A, Blanco-Méndez J, Martín-Pastor M. NMR techniques in drug delivery: application to zein protein complexes. Int J Pharm. 2012; 439(1-2): 41-48.

11. Farris E, Brown DM, Ramer-Tait AE, Pannier AK. Chitosan-zein nano-in-microparticles capable of mediating in vivo transgene expression following oral delivery. J Control Release. 2017; 249: 150-161.

12. Algahtani MS, Islam MS, Podaralla S, Kaushik RS, Reineke J, Woyengo T, et al. Food Protein Based Core-Shell Nanocarriers for Oral drug delivery: effect of shell composition on in vitro and in vivo functional performance of zein nanocarriers. Mol Pharm. 2017; 14(3): 757-769.

13. Jayaraman P, Sakharkar MK, Lim CS, Tang TH, Sakharkar KR. Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginora in vitro*. Int J Biol Sci. 2010; 6(6): 556-568.

14. Sarabhai S, Sharma P, Capalash. Ellagic acid derivates from *Terminalia chebula Retz.* Downregulate the Expression of quórum sensing genes to attenuate *Pseudomonas aeruginosa* PAO1 virulence. Plos one. 2013; 8(1): e53441.

15. Rúa J, Fernández-Álvarez L, Gutiérrez-Larraínzar M, Del Valle P, Arriaga D, García-Armesto R. Screening of phenolic antioxidants for their inhibitory activity against foodborne *Staphylococcus aureus* strains. Foodborne Pathog Dis. 2010; 7(6): 695-705.

16. Nayeem N, Karvekar. Antimicrobial and anti-oxidant properties of the isolated compounds from the methanolic extract from the leaves of *Tectona grandis*. J Basic Clin Pharm.2011; 2(4):163-165.

17. Al-Obaidi MM, Al-Bayaty FH, Al Batran R, Hassandarvish P, Rouhollahi E. Protective effect of ellagic acido on healing alveolar boné after tooth extraction in rat – A histological and immunohistochemical study. Arch Oral Bi*ol***.** 2014; 52(9): 987-999.

18. Gopalakrishnan L, Ramana LN, Sethuraman S, Krishnan UM. Ellagic acid encapsulated chitosan nanoparticles as anti-hemorrhagic agent. Carbohydr Polym. 2014; 111(13): 215-221.

19. Hermans K, Van den Plas D, Kerimova S, Carleer R, Adriaensens P, Weyenberg W, et al. Development and characterization of mucoadhesive chitosan films for ophthalmic delivery of cyclosporine A. Int J Pharm. 2014; 472(1-2):10-19.

20. Costa P, Sousa Lobo, JM. Modeling and comparison of dissolution profiles. Eur J Pharm Sci. 2001; 13(2): 123-133.

21. Ritger PL, Peppas NA. A simple equation for description of solute release I. Fickian and non-Fickian release from non-swellable devices in the form of labs, spheres, cylinders or discs. J Control Release. 1987; 5: 23-36.

22. Peppas NA, Sahlin JJ. A simple equation for the description of solute release. Coupling of diffusion and relaxation. Int J Pharm. 1989; 57: 169-172.

23. ASTM International. ASTM D882-02: standard test method for tensile properties of thin plastic sheeting. West Conshohochen: ASTM international, 2002.

24. Fundo JF, Fernandes R, Almeida PM, Carvalho A, Feio G, Silva CL, et al. Molecular mobility, composition and struture analysis in glycerol plasticised chitosan films. Food Chem. 2014; 144:2-8.

25. Morgado PI, Miguel SP, Correia IJ, Aguiar-Ricardo A. Ibuprofen loaded PVA/chitosan membranes: a highly eficiente strategy towards na improved skin wound healing. Carbohydr Polym. 2017; 159: 136-145.

26. Zhang J, Tan W, Zhang Z, Song Y, Li Q, Dong F, et al. Synthesis, characterization, and the antifungal activity of chitosan derivates containing urea groups. Int J Biol Macromol. 2017; 17: 33627-33633.

27. Santos TM, Souza Filho MSM, Muniz CR, Morais JPS, Kotzebue LRV, Pereira ALS, et al. Zein films with unoxidized or oxidized tannic acid. J Sci Food Agric. 2017; 97(13):4580-4587.

28. Yu H, Li W, Liu X, Li C, Ni H, Wang X, et al. Improvement of functionality after chitosan-modified zein biocomposites. J Biomater Sci Polym Ed. 2017; 28(3): 227-239.

29. Arockianathan PM, Sekar S, Kumaran B, Sastry TP. Preparation, characterization and evaluation of biocomposite films containing chitosan and sago starch impregnated with silver nanoparticles. Int J Biol Macromol. 2012; 50(4): 939-946.

30. Dashdorj U, Reyes MK, Unnithan AR, Tiwari AP, Tumurbaatar B, Parck CH, et al. Fabrication and characterization of electrospun zein/Ag nanocomposite mats for wound dressing applications. Int J Biol Macromol. 2015; 80:1-7.

31. Arulmozhi V, Pandian K, Mirunalini S. Ellagic acid encapsulated chitosan nanoparticles for drug delivery system in human oral cancer cell line (KB). Colloids Surf B Biointerfaces. 2013; 110: 313-320.

32. Ní Annaidh A, Bruyère K, Destrade M, Gilchrist MD, Otténio M. Characterization of the anisotropic mechanical properties of excised human skin. J Mech Behav Biomed Mater. 2012; 5(1): 139-138.

33.Ottenio M, Tran D, Ní Annaidh A, Gilchrist MD, Bruyère K. Strain rate and anisotropy effects on the tensile failure characteristics of human skin. J Mech Behav Biomed Mater. 2015; 41: 241-250.

34. Verma P, Ahuja M. Optimization, characterization and evaluation of chitosan-tailored cubic nanoparticles of clotrimazole. Int J Biol Macromol. 2015; 73: 138-145.

FIGURE LEGENDS

Figure 1 – Scanning electronic microscopy images of the films F1(A), F2(B), F3(C), F4(D), F5-F9(E), F10(F), F11(G), F12(H) and F13(I) obtained.

Figure 2 – FTIR spectra of chitosan (CHI), zein and ellagic acid (EA) individually.

Figure 3 – FTIR spectra of chitosan/zein films containing ellagic acid.

Figure 4 – *In vitro* release profilesof ellagic acid from CHI/zein films over 48 hours.

Figure 5 – Antimicrobial inhibitory activity of the CHI-zein loaded ellagic acid films against *Staphylococcus aureus*

Figure 6 – Antimicrobial inhibitory activity of CHI-zein loaded ellagic acid against *Pseudomonas aeruginosa*

TABELAS

Table I. Experimental design used

Films	CHI (X_1)		ZEI (X_2)	
F1	-1	0.199g	-1	0.199g
F2	-1	0.199g	+1	0.597g
F3	+1	0.597g	-1	0.199g
F4	+1	0.597g	+1	0.597g
F5-F9	0	0.398g	0	0.398g
F10	-√2	0.117g	0	0.398g
F11	+√2	0.679g	0	0.398g
F12	0	0.398g	-√2	0.117g
F13	0	0.398g	+√2	0.679g

CHI means chitosan (X_1). ZEI means zein (X_2).

Table II. Experimental results expressed as mean±SD for film thichness, sorption, solubility and angle contact of the obtained films

Films	Thickness (μm)	Sorption (%)	Solubility (%)	Angle contact (º)
F1	$150\pm54^{a.b}$	$163.44\pm24.27^{a.b.c.d}$	6.24 ± 0.98^{a}	70.23 ± 0.76^{a}
F2	$200\pm89^{a.b}$	$152.29\pm33.15^{a.b.c}$	7.54 ± 1.44^{a}	99.9 ± 0.62^{b}
F3	$216\pm75^{a.b}$	227.94 ± 25.88^{d}	5.21 ± 1.52^{a}	62.7 ± 0.62^{c}
F4	$266\pm81^{a.b}$	$167.73\pm24.61^{a.b.c.d}$	7.33 ± 1.47^{a}	76.46 ± 0.35^{e}
F5-F9	170 ± 74^{a}	$182.87\pm43.49^{b.c.d}$	6.86 ± 1.77^{a}	60.52 ± 0.83^{d}
F10	133 ± 51^{a}	$162.66\pm32.22^{a.b.c.d}$	12.65 ± 0.82^{b}	99.23 ± 0.73^{b}
F11	283 ± 75^{b}	$129.98\pm22.02^{a.b}$	16.46 ± 1.56^{c}	77.7 ± 0.45^{e}
F12	$183\pm75^{a.b}$	114.44 ± 8.07^{a}	11.16 ± 0.78^{b}	62.83 ± 0.35^{c}
F13	$200\pm89^{a.b}$	$217\pm40.05^{c.d}$	6.88 ± 1.57^{a}	98.56 ± 0.20^{b}

Same letter in the same column indicates that there is no significant difference between the mean values (Test of Turkey, $p<0,05$).

Table III. Kinect release models for *in vitro* EA release from the obtained films

Films	Zero ordem	Higuchi	Korsmeyer-Peppas		Peppas-sallin		
	R^2	R^2	R^2	n	R^2	K1	K2
F1	0.9923	0.9139	0.9971	1.20	0.9644	0.036	0.0378
F2	0.9858	0.9044	0.9955	1.29	0.9511	0.1710	0.1728
F3	0.9948	0.9634	0.9952	0.91	0.9734	0.0478	0.0493
F4	0.9891	0.9665	0.9928	0.84	0.9871	0.1283	0.1267
F5-F9	0.9986	0.9479	0.9984	1.01	0.9705	0.0284	0.0263
F10	0.9898	0.9440	0.9882	1.02	0.9624	0.4282	0.4256
F11	0.8668	0.9733	0.9760	0.33	0.9666	0.003	0.0071
F12	0.9872	0.9498	0.9852	0.97	0.9447	0.0371	0.0389

F13	0.9940	0.9195	0.9992	1.20	0.9687	0.1200	0.1225

Table IV. Mechanical properties of the chitosan/zein films F10 and F13.

	Tensile strength (MPa)	**Elongation at break (%)**
CHI	63.7 (±16.84)[a]	9.88 (±3.77)[a]
F10 without EA	8.99 (±3.5)[b]	3.04 (±0.63)[c]
F10 with EA	7.84 (±3.83) [b]	2.59 (±0.71)[c]
F13 without EA	8.82 (±4.65) [b]	7.95 (±2.61)[ab]
F13 with EA	10.03 (±6.03) [b]	5.16 (±1.83)[bc]

Values are expressed as mean±SD. Same letter in the same column indicates that there is no significant difference between the mean values (Test of Turkey, p<0,05).

Table V. Effect of chitosan (X_1) and zein (X_2) concentrations on the dependent variables: thickness, solubility, water uptake, contact angle, release of EA and antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

	X_1		**X_2**		**X_1X_2**		**X_1^2**		**X_2^2**	
	Factor effect	p-value	Factor effect	p-value	Factor effect	p-value	Factor effect	p-value	Factor effect	p-value
Thickness	86.08	<0.001	31.04	0.032	0.00	1.00	42.81	0.011	26.2	0.073
Solubility	1.11	0.658	-0.73	0.770	0.56	0.874	5.05	0.091	-0.49	0.853
Water uptake	8.46	0.807	18.39	0.60	-24.6	0.62	-25.6	0.499	-6.19	0.868
Contact angle	-15.36	0.001	23.5	<0.001	-7.95	0.091	24.34	<0.001	16.55	<0.001
Release of EA	-1.21	0.073	2.30	0.005	-0.46	0.588	-0.53	0.41	0.51	0.432
A.A S.aureus	4.10^5	0.128	-1.10^5	0.643	4.10^5	0.339	-4.10^5	0.231	-3.10^5	0.282
A.A P.aeruginosa	2.10^7	0.056	-6.10^5	0.957	1.10^7	0.50	1.10^7	0.22	5.10^6	0.69

A.A = Antimicrobial activity

FIGURES

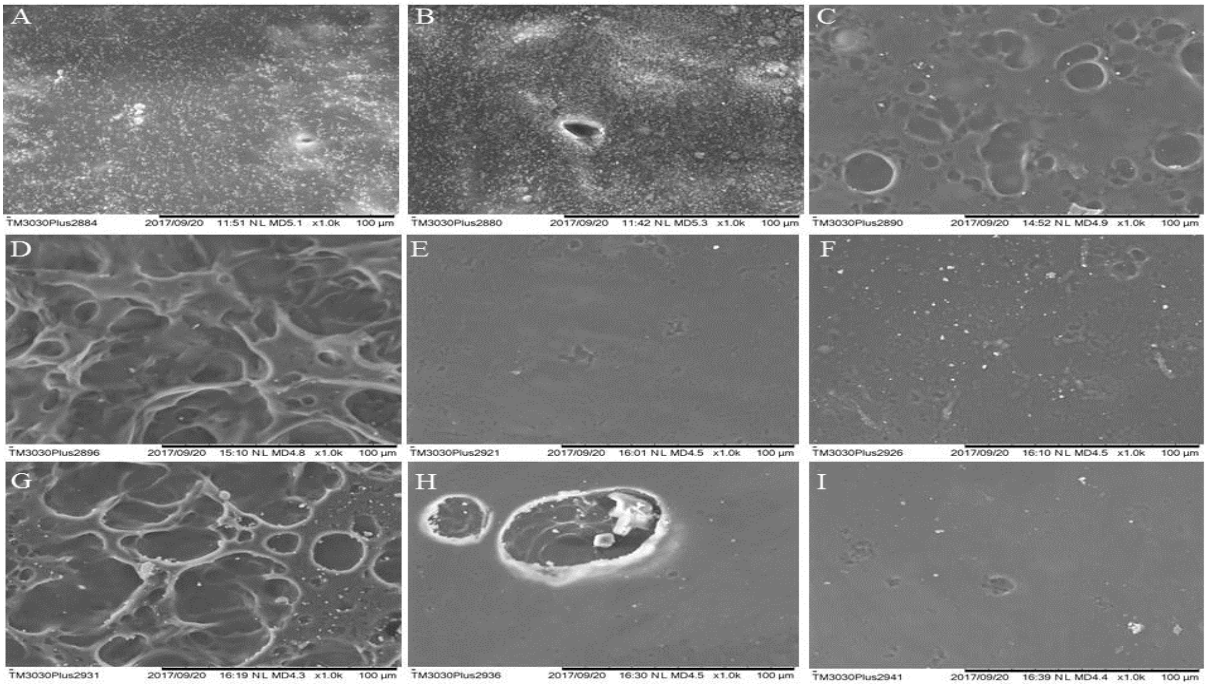

Figure 1 – Scanning electronic microscopy images of the films F1(A), F2(B), F3(C), F4(D), F5-F9(E), F10(F), F11(G), F12(H) and F13(I) obtained.

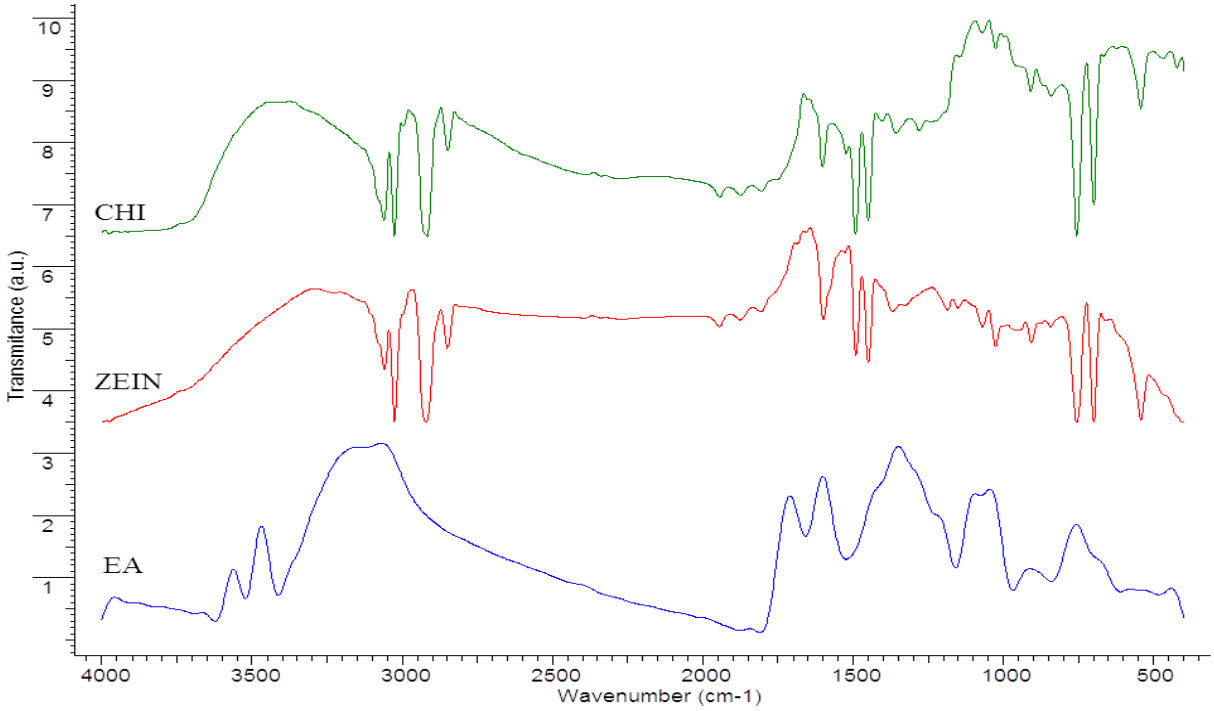

Figure 2 – FTIR spectra of chitosan (CHI), zein and ellagic acid (EA) individually.

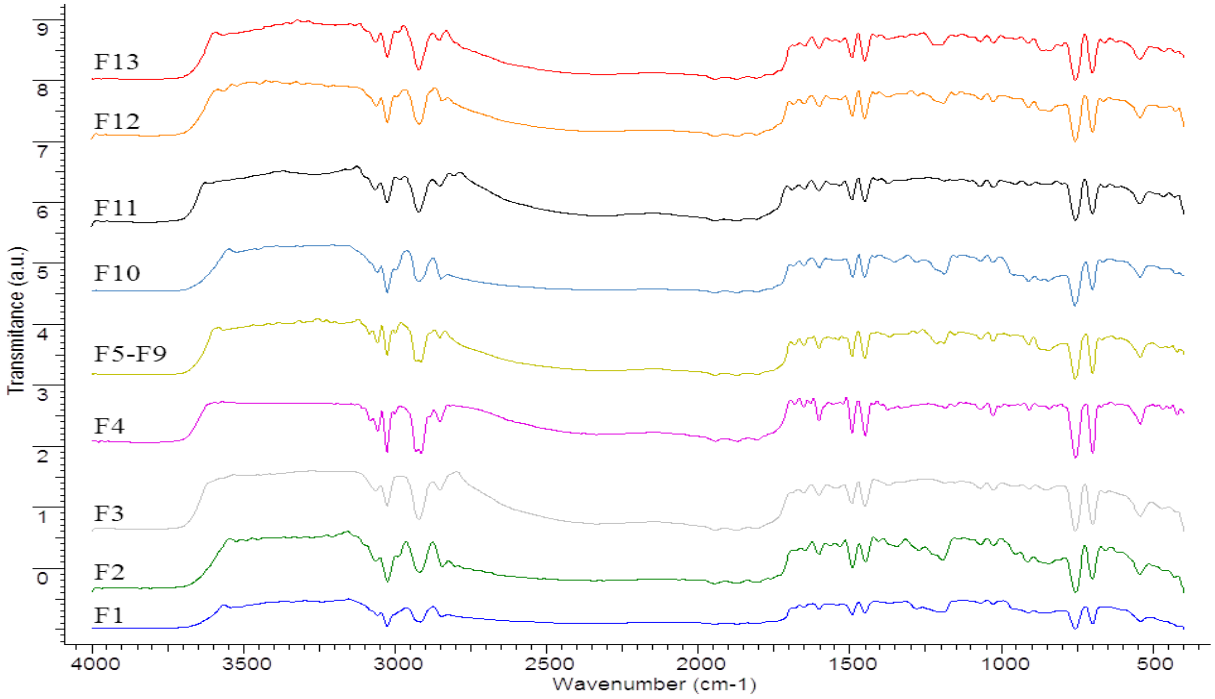

Figure 3 – FTIR spectra of chitosan/zein films containing ellagic acid.

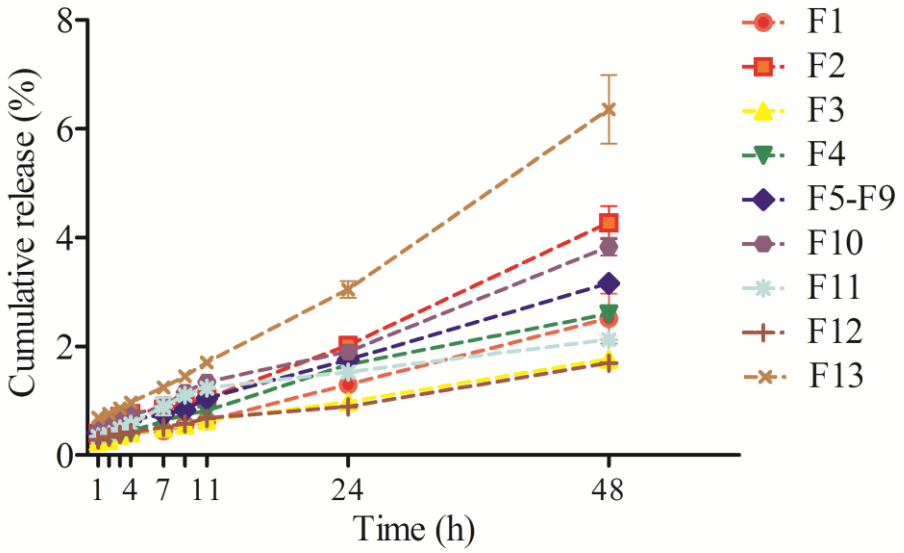

Figure 4 – *In vitro* release profilesof ellagic acid from CHI/zein films over 48 hours.

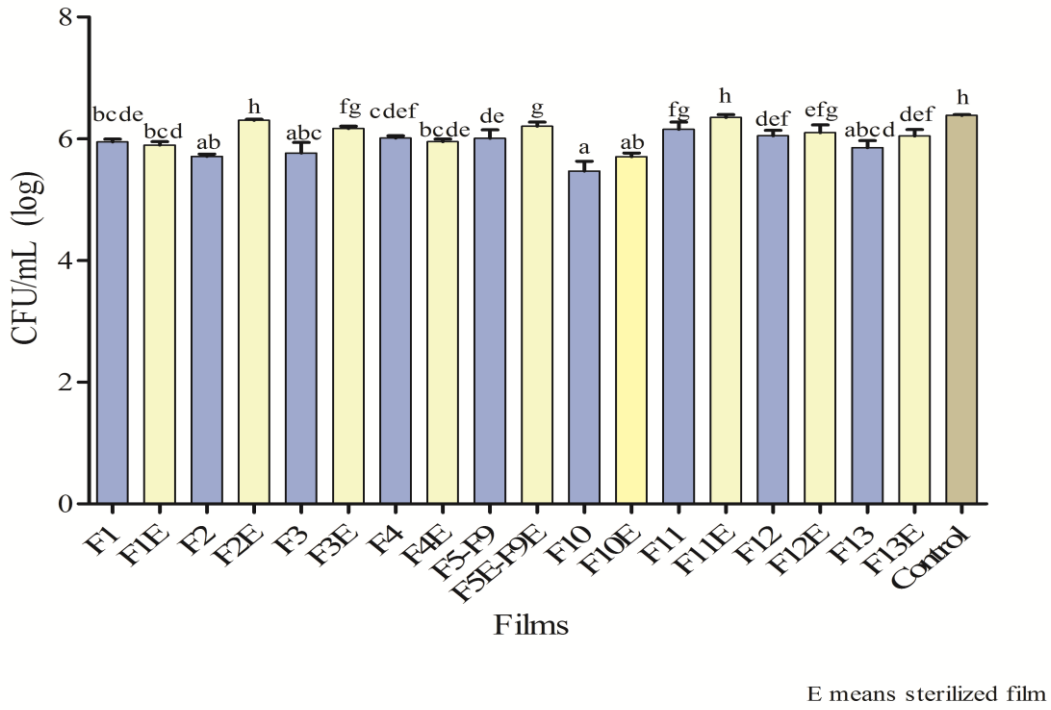

Figure 5 – Antimicrobial inhibitory activity of the CHI-zein loaded ellagic acid films against *Staphylococcus aureus*

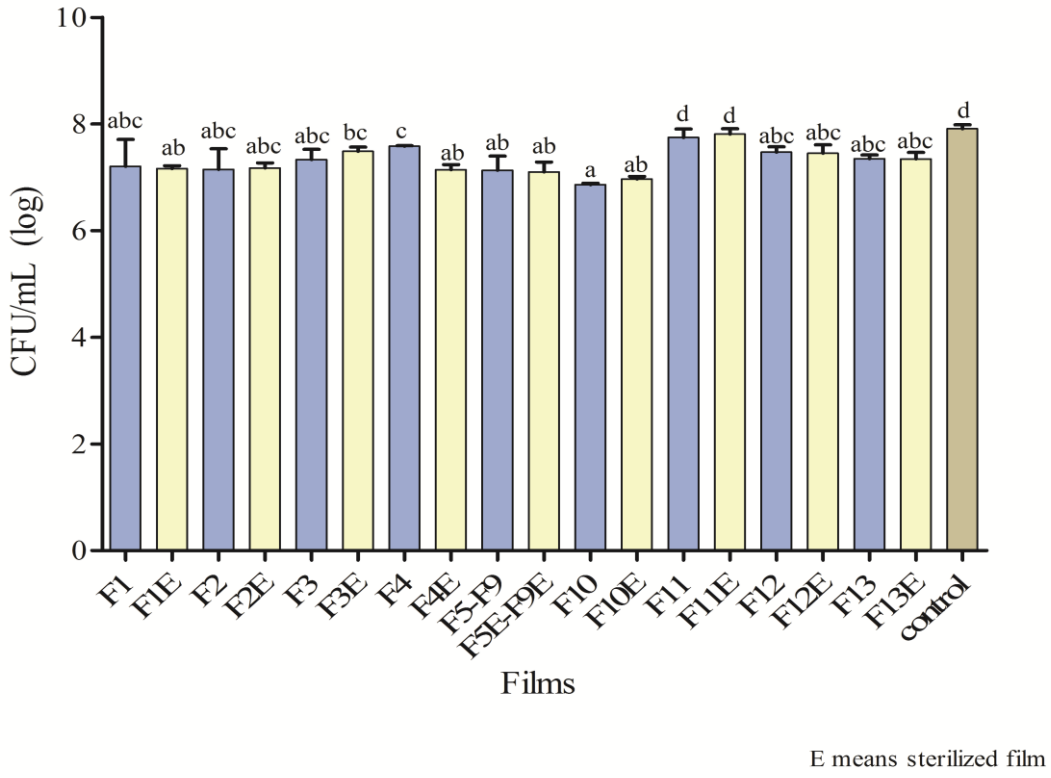

Figure 6 – Antimicrobial inhibitory activity of CHI-zein loaded ellagic acid against *Pseudomonas aeruginosa.*

6 CONSIDERAÇÕES FINAIS

- Os valores obtidos para o MIC e MBC do EA para *Staphylococcus aureus* foram 0,104 e 0,291 mg/ml e para *Pseudomonas aeruginosa* 0,208 e 0,416 mg/ml, respectivamente, ratificando a capacidade antimicrobiana do EA.
- A zeina potencializou o efeito antimicrobiano do EA contra *P.aeruginosa* e a CHI potencializou a ação inibitória do EA tanto frente a *S.aureus* quanto a *P.aeruginosa.*
- A zeina e a CHI, testadas sozinhas, também apresentaram atividade antimicrobiana frente a ambas bacterias*.*
- O EA apresentou atividade antioxidante com IC_{50} de 0,079 mg/ml. Exibiu ainda atividade inibitória frente a colagenase e elastase de 66,83% e 48,34%, respectivamente.
- A zeina sozinha na concentração de 2,5 mg/ml apresentou atividade antioxidante acima de 35% e também melhorou a atividade antioxidante do EA em concentrações abaixo de 0,125mg/ml.
- Os filmes de CHI e zeina contendo EA foram desenvolvidos e satisfatoriamente caracterizados utilizando o modelo de desenho experimental proposto.
- Foi confirmada através dos resultados de RMN a interação química existente entre o EA e a zeína, o que seria um fator crucial para a funcinalidade das membranas.
- Os filmes obtidos mostraram características físicas e biológicas favoráveis para seu uso no tratamento de feridas e/ou outras condições biomédicas nas que as propriedades farmacológicas do EA possam ser benéficas.
- O filme F13 foi o que apresentou as melhores respostas: espessura adequada, alta sorção, baixa solubilidade, superfície hidrofílica, lisa e densa, melhor perfil

de liberação (lenta e controlada), capacidade inibitória contra as bactérias testadas, força tênsil e alongamento adequados, sendo, portanto, um candidato promissor para pesquisas futuras.

REFERÊNCIAS

AL-OBAIDI, M.M.; AL-BAYATY, F.H.; AL BATRAN, R.; HASSANDARVISH, P.; ROUHOLLAHI, E. Protective effect of ellagic acido n healing alveolar boné after tooth extraction in rat – A histological and immunohistochemical study. **Archives of Oral Biology,** v. 52, n. 9, p. 987-999, 2014.

BESERRA, A.M.; CALEGARI, P.I.; SOUZA, M.C.; SANTOS, R.A.; LIMA, J.C.; SILVA, R.M.; BALOGUN, S.O.; MARTINS, D.T. Gastroprotective and ulcer-healing mechanisms of ellagic acid in experimental rats. **Journal of Agricultural and Food Chemistry,** v. 59, n. 13, p. 6957-6965, 2011.

BIZERRA, A.; SILVA, V. Sistemas de liberação controlada: mecanismos e aplicações. **Revista saúde e meio ambiente,** v. 3, n. 2, p. 1-12, 2016.

CHATTERJEE, A.; CHATTERJEE, S.; DAS, S.; SAHA, A.; CHATTOPADHYAY, S.; BANDYOPADHYAY, S.K. Ellagic acid facilitates indomethacin-induced gastric ulcer healing via COX-2 up-regulation. **Acta Biochimica et Biophysysica Sinica,** v. 44, p. 565-576, 2012.

ESCAMILLA-GARCIA, M.; CALDERÓN-DOMINGUES, G.; CHANONA-PÉREZ, J.J.; FARRERA-REBOLLO, R.R.; ANDRACA-ADAME, J.A.; ARZATE-VÁSQUEZ, I.; MENDEZ-MENDEZ, J.V.; MORENO-RUIZ, L.A. Physical and structural characterisation of zein and chitosan edible films using nanotechnology tools. **International Journal of Biological Macromolecules,** v. 61, p. 196-203, 2013.

GARCÍA-NIÑO, W.R.; ZAZUETA, C. Ellagic acid: pharmacological activities and molecular mechanisms involved in liver protection. **Pharmacological Research,** v. 97, p. 84-103, 2015.

GOPALAKRISHNAN, L.; RAMANA, L.N.; SETHURAMAN, S.; KRISHNAN, U.M. Ellagic acid encapsulated chitosan nanoparticles as anti-hemorrhagic agent. **Carbohydrate Polymers,** v. 111, n. 13, p. 215-221, 2014.

HELANDER, I.M.; NURMIAHO-LASSILA, E.L.; AHVENAINEN, R.; RHOADES, J.; ROLLER, S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. **International Journal of Food Microbiology,** v. 71, p. 235-244, 2001.

KIM, J.O.; NOH, J.K.; THAPA, R.K.; HASAN, N.; CHOI, M.; KIM, J.H.; et al. Nitric oxide-releasing chitosan film for enhanced antibacterial and *in vivo* wound-healing efficacy. **International Journal of Biological Macromolecules,** v. 79, p. 217-225, 2015.

NAYEEM, N.; KARVEKAR, M. Anti microbial and anti-oxidant properties of the isolated compounds from the methanolic extract from the leaves of *Tectona grandis*. **Journal of Basic and Clinical Pharmacy,** v.2, n. 4, p. 163-165, 2011.

OKAMOTO, Y.; KAWAKAMI, K.; MIYATAKE, K.; MORIMOTO, M.; SHIGENASA, Y.; MINAMI, S. Analgesic effects of chitin and chitosan. **Carbohydrate Polymers,** v. 49, p. 249-252, 2002.

OKAMOTO, Y.; YANO, R.; MIYATAKE, K.; TOMOHIRO, I.; SHIGENASA, Y.; MINAMI, S. Effects of chitin and chitosan on blood coagulation. **Carbohydrate Polymers,** v. 53, p. 337-342, 2003.

OLIVEIRA, M.R. The effects of Ellagic acid upon brain cells: a mechanistic view and future directions. **Neurochemical Research.,** v. 41, p. 1219-1228, 2016.

SOLIMAN, E.A.; KHALIL, A.A.; DERAZ, S.F.; EL-FAWAL, G.; ABD ELRAHMAN, S. Synthesis, characterization and antibacterial activity of biodegradable films prepared from Schiff bases of zein. **Journal Food Science Technology,** v. 51, n. 10, p 2425-2434, 2014.

TANG, N.; ZHUANG, H. Evaluation of antioxidante activities of zein protein fractions. **Journal of Food Science,** v. 79, n. 11, p. 2174-2184, 2014.

XING, R.; LIU, S.; GUO, Z.; YU, H.; WANG, P.; LI, C.; et al. Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities *in vitro*. **Bioorganic and medicinal chemistry,** v. 13, p. 1573-1577, 2005.

Anexo 1 – Normas de publicação da Revista Polímeros

Instructions to Authors

Aims:

"Polímeros" edited by the Brazilian Polymer Association (Associação Brasileira de Polímeros - ABPol) aims to disseminate worldwide the scientific knowledge developed in the polymer science and technology field.

Policy

The submission of a paper implies that it has not been previously published, that it is not under consideration for publication elsewhere, and that it will not be published elsewhere in the same form without the written permission of the Editors. By submitting a manuscript, the authors agree that the copyright for their article is transferred to Polímeros: Ciência e Tecnologia if and when the article is accepted for publication. Accepted articles and illustrations become the property of Polímeros: Ciência e Tecnologia.

Types of articles:

Polímeros publishes Review Articles, Original Articles and Short Communications in the polymer area.

Review Articles: These manuscripts should deals and discuss deeply and critically the available and up-dated scientific knowledge in a particular subject. Should be submitted only by senior researchers/professionals who have done research and published articles (in indexed journals of international acceptance) on the presented subject. These publications have to be included in the references list. Articles which are only a bibliographic review, not containing a critical analysis of the author(s), will not be accepted.

Original Articles: These manuscripts refer to original works with unpublished results, showing real progress and significant contribution to the field of polymers.

Short Communication: These manuscripts are compact works dealing with recent developments, which results the authors consider should be focusing directly on results/original proposals. Because of their nature, these communications will go through a “fast track” review and publication process.

Presentation:

The manuscript is to be written in English, and great care should be taken for its objectiveness, clarity, and conciseness. Authors are responsible for having the manuscripts reviewed by a native English speakers or professionals with solid knowledge of technical language. This has to be formally confirmed in an accompanying letter stating that spelling and text structure were reviewed. Please refer to the *Template* provided for its formatting.

Length of manuscripts:

All manuscripts (which include text, figures, tables, photos, etc.) have a limited number of pages according to its type: Review Articles are limited to a maximum of 30 pages; Original Articles must not exceed 20 pages, whileShort Communications must not exceed 8 pages. Note that these page limitations where set formatting the manuscript according to: A4 format page, with 25 double-spaced lines, typed in font "Times New Roman" size 12.

Text, Figures and tables:

Please refer to *Template* for further information in how to present and format your contribution.

References:

Please refer to the *Template* and follow carefully the way the references should be presented. Refer to the examples provided. Please avoid refer to texts with are not easily found, with only local dissemination, written in languages other than English. Theses and dissertations which comply with these cases should be avoided. Monographs and similar texts must NOT be used as references.

Online submission and evaluation:

All manuscripts submitted for publication will be evaluated by the Editorial Board of the Journal, with the help of AdHoc reviewers, which are experts in the technical subject of the manuscript. Manuscripts must strictly follow the guidelines for publication, otherwise they will be returned to their authors. The approval is subject to their technical quality which is substantiated by the reviewers' opinion. The submission should be done electronically within the submission system available at https://mc04.manuscriptcentral.com/po-scielo. Only one of the authors should submit the manuscript, and for that he will have to register his e-mail and will become the corresponding author. Any communication between the editorial board and the authors will take place through this registered e-mail. **The online submission and evaluation steps are free of charge.**

Article-charge:

The Editorial Board of Polímeros has decided to apply an "article-charge" to all accepted articles in order to have it published. When submitting a manuscript, the submitting author will be asked to confirm his knowledge and acceptance for this cost. The value is fixed per article, independent of its length and is R$800,00 (in Brazilian reais) or U$230,00 (in American dollars).

Revised version

It is compulsory, when submitting the revised version, to add a letter "Response to Reviewers" in which the AdHoc reviewers' comments and questions are individually and thoroughly addressed. In it the authors must inform the number of the page/paragraph in which changes/additions/removal have been made. In case authors do not agree with any comments and/or requests of the reviewers, they may rebuttal explaining and justifying their understanding of the matter in the letter. Any change done in the revised version must be highlighted with a color other than black.

Final decision

The Editorial Board of **Polímeros** is responsible of accepting/rejecting any manuscript submitted to this journal. Their decision is final, and NO appeal can be enforced.

Anexo 2 - Normas para submissão da *International Journal of Pharmaceutics*

Instructions to Authors

Use of word processing software It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately

after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

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Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.
The abstract must not exceed 200 words.
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A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more, but should be readable on screen at a size of 200 × 500 pixels (at 96 dpi this corresponds to 5 × 13 cm). Bear in mind readability after reduction, especially if using one of the figures from the article itself. Preferred file types: TIFF, EPS, PDF or MS Office files.
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Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

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Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:
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If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

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Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

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2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

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Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Reference to a website:
Cancer Research UK, 1975. Cancer statistics reports for the UK.
http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).

Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oak wilt disease and surrounding forest compositions. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.

Anexo 3 - Normas para submissão da *Journal of Biomedical Materials Research* Part A

Instructions to Authors

Author Guidelines

Journal of Biomedical Materials Research Part A

Information for Contributors

Aims and Scope

The Journal of Biomedical Materials Research Part A is an international, interdisciplinary, English-language publication of original contributions concerning studies of the preparation, performance, and evaluation of biomaterials; the chemical, physical, toxicological, and mechanical behavior of materials in physiological environments; and the response of blood and tissues to biomaterials. The Journal publishes peer-reviewed articles on all relevant biomaterial topics including the science and technology of alloys,polymers, ceramics, and reprocessed animal and human tissues in surgery,dentistry, artificial organs, and other medical devices. The Journal also publishes articles in interdisciplinary areas such as tissue engineering and controlled release technology where biomaterials play a significant role in the performance of the medical device.

The Journal of Biomedical Materials Research is the official journal of the Society for Biomaterials (USA), the Japanese Society for Biomaterials, the Australasian Society for Biomaterials, and the Korean Society for Biomaterials.

Use of Animals: When animals are used in the research reported,the authors must state: "NIH guidelines (or for non-U.S. residents similar national regulations) for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed." In studies involving human subjects, the authors must include; "all subjects enrolled in this research have responded to an Informed Consent which has been approved by my Institutional Committee on Human Research and that this protocol has been found acceptable by them." The dates of approval by the Committee and the ethical guidelines followed should be made a part of the Methods section.

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1.1.1.1 Instructions for Manuscript Preparation

Manuscript: For optimal production, prepare manuscript text in size 12 font on 8-1/2 x 11 inch page, double-spaced, with at least 1-inch margins on all sides. Text files should be formatted as .doc or .rtf files**.** Refrain from complex

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References:
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1. King VM, Armstrong DM, Apps R, Trott JR. Numerical aspects of pontine, lateral reticular, and inferior olivary projections to two paravermal cortical zones of the cat cerebellum. J Comp Neurol 1998;390:537-551.

2. Voet D, Voet JG. Biochemistry. New York: John Wiley & Sons; 1990. 1223 p.

3. Gilmor ML, Rouse ST, Heilman CJ, Nash NR, Levey AI. Receptor fusion proteins and analysis. In: Ariano MA, editor. Receptor localization. New York: Wiley-Liss; 1998. p 75-90. Please note that journal title abbreviations should conform to the practices of Chemical Abstracts.

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Illustrations: When preparing digital art, please consider:
Resolution: The minimum requirements for resolution are:
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