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PRODUCTION AND CHARACTERIZATION OF NANOCAPSULES OF GERANIL CINNAMATE AND ITS EVALUATION AS POSSIBLE ANTIMICROBIAL ADDITIVE

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Doctorate thesis for the degree of Doctor in Chemical Engineering presented to the Graduate Program in Chemical Engineering at Federal University of Santa Catarina.
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Micheli Zanetti

PRODUCTION AND CHARACTERIZATION OF NANOPARTICLES OF GERANYL CINNAMATE AND POLICAPROLACTONE POLYMER

This thesis was considered adequate for the degree of Doctor in Chemical Engineering and was approved in its final form by the Graduate Program in Chemical Engineering at Federal University of Santa Catarina.

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"The true motivation comes from achievement,
personal development, satisfaction
in work and recognition "

Unknown author

ABSTRACT

Essential oils present new opportunities for solutions for the food industry. These are complex mixtures of a variety of volatile molecules that can easily be decomposed, depending on the industrial application. The geraniol is a terpenic alcohol found in many essential oils of herbs and their antimicrobial property increases its use as an additive for the food industry. However, the use of its beneficial effects is limited due to their instability opposite to environmental chemical degradation caused by oxidation and temperature. Through an experimental design the reaction was studied of geraniol and cinnamic acid to obtain geranyl cinnamate ester which was obtained at a conversion of 97% under the conditions of 80 °C reaction, by *Candida Antarctica* immobilized NS88011 20% weight, and molar ratio of geraniol and cinnamic acid 5:1. The antimicrobial activity of geranyl cinnamate ester was tested on *Staphylococcus aureus* and *Escherichia coli* and three types of fungi: yeast *Candida albicans, Penicillium variabile* and *Aspergillus niger*. The agar diffusion test compound showed excellent antimicrobial activity for all the microorganisms. The nanoencapsulation can be an alternative to protect the cinnamate geranyl ester of an unfavorable environment, and to promote a controlled release of this compound. For this, nanocapsules with geranyl cinnamate ester polymer and polycaprolactone (PCL) were obtained by nanoprecipitation technique with solvent evaporation. The results show that nanocapsules with an average size of 177.6 nm and a polydispersity index of less than 0.200 were obtained, showing the formation of nanocapsules with a homogeneous size. Electron microscopy results showed the formation of spherical nanocapsules and the TGA results showed the thermal protection by the PCL coating of the ester, and the encapsulation increased the temperature for the release of the geranyl cinnamate ester at 20 ºC. Aqueous dispersions of geranyl cinnamate loaded PCL nanoparticles stored at 4ºC presented good colloidal stability over 60 days. Minimum inhibitory concentration (MIC) tests showed that the geranyl cinnamate was not released from the PCL nanoparticles in aqueous solution even after 72 hours, requiring the use of a trigger (oil phase, lipase to degrade the polymer matrix e.g) to release the active compound. Tests have shown that raising the temperature in industrial processes can be a trigger for releasing the active compound from within the capsules. Acute systemic toxicity tests in mice showed that the geranyl cinnamate ester is non-toxic up to the concentration of 300 mg kg^{-1}.

Keywords: ester geranyl cinnamate, antimicrobial, nanoencapsulation, additive.

RESUMO

Óleos essenciais têm apresentado novas perspectivas de soluções para a indústria de alimentos. Estes são misturas complexas de uma grande variedade de moléculas voláteis que podem ser facilmente decompostas, dependendo da aplicação industrial. O geraniol é um álcool terpênico encontrado em muitos óleos essenciais de plantas aromáticas e sua propriedade antimicrobiana aumenta sua utilização como aditivo para a indústria alimentar. No entanto, a utilização de seus efeitos benéficos é limitada devido à sua instabilidade frente às degradações químicas do ambiente, causadas pela oxidação e temperatura. Através de um planejamento experimental foi estudada a reação do geraniol e o ácido cinâmico para obtenção do éster geranil cinamato, o qual foi obtido numa conversão de 97% nas condições de reação de 80 °C, 20% em peso de *Candida antarctica* NS88011 imobilizada, e para proporção molar de geraniol e ácido cinâmico de 5:1. A atividade antimicrobiana do éster geranil cinamato foi testada frente as bactérias *Staphylococcus aureus* e *Escherichia coli* e três tipos de fungos: levedura *Candida albicans*, *Penicillium variabile* e *Aspergillus niger*. Pelo teste de difusão em ágar o composto mostrou uma excelente atividade antimicrobiana para todos os microrganismos testados. A nanoencapsulação pode ser uma alternativa para proteger o éster geranil cinamato de um ambiente desfavorável, além de promover uma liberação controlada deste composto. Para isso, nanocápsulas poliméricas de éster de geranil cinamato e policaprolactona (PCL) foram obtidas pela técnica de nanoprecipitação com a evaporação do solvente. Os resultados mostraram que nanocápsulas com tamanho médio de 177,6 nm e índice de polidispersão menor que 0,200 foram obtidas, mostrando a formação de nanocápsulas com tamanho homogêneo. Os resultados de microscopia de transmissão eletrônica (TEM) mostraram a formação de nanoparticulas esféricas e os resultados de TGA mostraram a proteção térmica pelo revestimento de PCL do éster, sendo que a encapsulação aumentou a temperatura para a liberação do éster de geranil cinamato em 20 ^{o}C. Dispersões aquosas de nanopartículas de PCL carregadas com geranil cinamato armazenadas a 4 ^{o}C apresentaram boa estabilidade coloidal ao longo de 60 dias. Os testes de Concentração Inibitória Mínima (CIM) mostraram que o geranil cinamato não foi liberado das nanopartículas de PCL em solução aquosa, mesmo após 72 horas, exigindo o uso de um gatilho (fase oleosa, lipase para degradar a matriz polimérica, por exemplo) para liberar o composto ativo. Testes mostraram que temperaturas elevadas em processos industriais pode ser um gatilho para liberar o composto ativo de dentro das cápsulas.

Testes de toxicidade sistêmica aguda em camundongos mostraram que o éster de geranil cinamato não é tóxico até a concentração de 300 mg kg^{-1}.

Palavras-chave: Éster geranil cinamato, antimicrobiano, nanoencapsulação, aditivo.

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SUMMARY

1 WHAT IS THIS THESIS ABOUT?

Synthetic food chemical preservatives can be harmful to human health and provide numerous serious gastric problems. These indications have increasingly aroused the concern and interest of researchers in studies and applications of natural compounds with this function in food. The use of natural antimicrobial compounds in food has been observed by most consumers and the food industry.

The use of preservatives in industrialized foods is necessary and widely used by the food industry. These compounds help to control the growth of bacteria and fungi. However, many studies indicate that these can cause cancer and allergies in humans (Janjarasskul et al., 2016). Despite health hazards, the truth is that these preservatives are essential to prolong the storage time of food. In this scenario, the use of natural antimicrobials is an attractive option.

Natural antimicrobials can be obtained from different sources including plants, animals, bacteria, algae and fungi. Several studies on antimicrobials obtained from plants have demonstrated the efficacy of these compounds when applied for food preservation, and have also investigated the factors, such as forms of exposure, that influence the effectiveness of this protection (Gyawali and Ibrahim, 2012; Hayek, Gyawali and Ibrahim, 2013; Tajkarimi, Ibrahim and Cliver, 2010).

In this sense, several studies have evaluated the efficacy of essential oils derived from oleaginous plants, for example thymol, carvacrol, allicin, geraniol, limonene, among others. These essential oils have been shown to inhibit the growth of bacterial pathogens of food origin (Ozogul, et al., 2015).

Phenolic compounds and other hydrophobic compounds present in essential oils attribute these plant extracts to antimicrobial activity (Dorman and Deans, 2000). Therefore, essential oils have been suggested as alternative sources for antimicrobial treatments (Singh et al., 2009; Zabka et al., 2009; Kumar et al., 2010). Natural phenolic substances, found in plant essential oils, are among the most active antifungal substances. Despite the antifungal, antibacterial and high insecticidal efficacy, these substances have a low toxic effect in humans (Xu et al., 2008; Ahmad et al., 2011).

The essential oils interact with the cells of the microorganisms breaking the plasma membrane and damaging the cytoplasm (Hosein et al. 2011). In certain situations, the compounds completely alter the morphology of the cells and cause them to die (Maillard, 2002). In particular, geraniol essential oil has a high inhibiotion capacity of Gram-

positive and Gram-negative bacteria, as well as some types of fungi and some types of yeasts (Zanetti et al, 2015).

In the same sense of obtaining natural compounds with food protection capacity many cinnamic acid derivatives, especially those containing the phenolic hydroxyl group, have been investigated. Cinnamic acid derivatives are antioxidants known to provide a number of benefits to human health because of their high ability to eliminate free radicals. Cinnamic acid derivatives are also recognized and cited for their antimicrobial properties (Dorman and Deans, 2000).

Esters derived from cinnamic acid, for example, because they have antioxidant and antimicrobial properties, besides being important aromas used in the food industry, have aroused interest in both the scientific and industrial community (Dôres, 2007). Geranyl cinamate, although little known in the literature, presents indicative of antimicrobial and antioxidant properties (Pankaj et al., 2013).

The esterification and transesterification reactions, common in industry, are being increasingly studied in biotechnological processes, using enzymatic routes. In spite of the scientific and technological importance for these esters, there are few detailed studies reporting the routes of obtaining and evaluating the effect of the parameters of the synthesis processes of the geranyl cinnamate ester. No published study provides detailed information on the antimicrobial and antioxidant properties of these compounds. Therefore, the need for detailed studies of the enzyme catalyzed production of these compounds from the esterification of geraniol and cinnamic acid, as well as their antimicrobial properties, is an eminent necessity and opportunity.

In order to protect essential oils, which are very volatile, thermo-degradation and photodegradation factors, nanoencapsulation has been an important procedure. It is a technique in which solid, liquid and gaseous substances, compounds or agents are coated with an encapsulating agent, there by obtaining nanometer-sized particulates. Nanoencapsulation consists of the compartmentalization of substances in carriers, whose typical size is 50 to 300 nm. Encapsulation is an interesting and important procedure for increasing the physical-chemical and microbiological stability, for the physical and chemical protection of the agents, and also for allowing the controlled release of the encapsulated substance.

The rapid advance in the nanotechnology area of the last decades has boosted the development of advanced techniques for the release of active compounds in the most diverse processes and sectors (Donsi et al., 2011). The production of polymer nanocapsules has been the focus of pharmaceutical and food industries as well as the scientific community

since they have interesting characteristics and properties when used as controlled release systems for active compounds (Brandelli and Taylor, 2015).

Many ongoing studies investigate the use of nanotechnology in food and supplement packaging, with the application of nanomaterials (Mozafari et al., 2008). Studies in the last years have shown that the encapsulation of essential oils in nano-sized capsules can be used to protect the active compounds against environmental factors such as oxygen, light, moisture and pH (Donsi et al. 2011; Gortzi et al., 2007 and Liolios et al., 2009).

The encapsulation of eugenol, thymol and carvacrol in nanometric surfactant micelles also resulted in improved antimicrobial activity (Gaysinsky et al., 2005, Chen, Zhang and Zhong, 2015, Castillo et al., 2014 and Pan et al., 2014). This result was also observed in studies that encapsulated *Origanum Dictamnus L*. (Liolios et al., 2009), Citrus limon extracts (Gortzi et al., 2007), *Myrtus* extracts (Gortzi et al., 2008) of essential oil *Artemisia arborescens L*. (Sinico et al., 2005).

It is verified that the nanoencapsulation already shows signs of dissemination of the technique, however its use in industrial products is still in its initial stages. Different alternatives can be provided for packaging, such as the development of nanoparticles, nanodispersions, nanolamines and nanotubes which, associated with polymers, can provide several functions.

For example, by incorporating an active natural agent into a nanocapsule, it becomes a capsule with antimicrobial property, in addition to being able to keep the compounds under optimum conditions for their preservation, the capsule being a barrier to moisture, oxygen and absorption of UV irradiation, as well as increase the stability of the active compounds against temperature.

In relation to the physical properties of the esters, it is known that these have high volatility, which is confirmed these characteristic aromas. However, its specific application in active packaging is limited, since the esters may be poorly stable under the conditions of operation of a process for obtaining plastic films, for example, which use high temperatures; or for the use of this package in a food, in such conditions that (pH, light exposure) can cause the ester to undergo hydrolysis or oxidation. Accordingly, ester protection via polymer encapsulation becomes an interesting alternative because it protects the compound from the operating conditions and environmental conditions by releasing its active ingredients at the desired time and conditions.

Polycaprolactone (PCL) has received much attention as an ideal material for controlled release of drugs and other applications (Mondrinos et al., 2006). The poly-ε-caprolactone (PCL) is an excellent polymer for the encapsulation of natural extracts and essential oils for food applications as antimicrobial additive (Zambrano-Zaragoza et al., 2011). The PCL is a somewhat hydrophobic and semicrystalline polymer with high molecular weight that may be used in diffusion-controlled delivery systems (Marchal-Heussler et al., 1992). Polycaprolactone, is a semicrystalline aliphatic polyester, considered as a biocompatible, and bioresorbable material with high permeability to drugs. The PCL is approved by the FDA for food applications (Nair and Laurencin, 2007; Hutmacher et al., 2001). This polymer also has a low glass-transition temperature of -60 °C, a melting point of 60 °C and exhibits high decomposition temperature around 350 °C.

The hydrolytic stability of their ester linkages renders the PCL biodegradability under physiological conditions. Due to its high degree of crystallinity and hydrophobicity, it degrades slowly and this may be interesting in the case of a release of a microbiologically active compound, applied to control microbial growth in food packaging. In relation to PCL nanocapsules, the characteristics of size, shape and zeta potential, strongly influence the degradation and release of the active compounds (Grossen et al, 2017). Moreover, encapsulation techniques using PCL have already improved the heat stability of the active compounds (González-Reza et al., 2015).

PCL was used by the authors Sosa et al. (2011) to microencapsulate green tea polyphenols and 3-5 μm microcapsules were obtained. The results of the drug release showed that it was released progressively in 90 h. The authors suggested that in the PCL, a rapid initial release occurs due to the diffusion of the drug through the matrix, and the rest of the active compost is released only when the matrix begins to degrade. Limayem Blouza et al. (2006) also encapsulated olive oil with PCL, obtaining capsules with a nanometer size of 320 to 400 nm.

The paper and polymer packaging industry faces the challenge of selling and distributing food products with the largest possible storage periods, with a view to increasing the distribution and storage period (Braga and Peres, 2010). Likewise, with the commitment of food safety to consumers. Thus, the concept of active packaging has emerged, which not only promotes an inert barrier to external influences, but also interacts with the product and has important properties for food protection and consumer food safety.

This segment seeks new technologies to develop innovative packaging that has antimicrobial and antioxidant properties, protecting the food from environmental conditions and consequently guaranteeing the health of the final consumer. Added to this, it is known that many of the additives currently used in food preservation are toxic to humans and need to be used with concentration restriction in accordance with current legislation. Therefore, the use of geranyl cinnamate nanocapsules as a natural antimicrobial additive in packaging presents itself as an opportunity for the sector, since they are non-toxic products and can be protected from environmental and process factors due to encapsulation. In addition, it can allow an increase in shelf-life of these products, bringing a significant gain to the industries, making this an interesting research financially for this industry.

Thus, this work presents the nanoencapsulation of the geranyl cinnamate ester it is possible to obtain a natural antimicrobial compound with greater thermal stability and with this to potentiate it as a possible antimicrobial additive nontoxic for application in food packaging. For this, in a first procedure the geranyl cinnamate ester was synthesized from enzymatic esterification of geraniol and cinnamic acid. The compound was characterized as its physical, chemical and microbiological properties. In a second procedure the feasibility of nanoencapsulation of the geranyl cinnamate ester using the PCL polymer as the encapsulating agent was studied and the effects of some parameters of the encapsulation process, such as temperature and chemical composition of the reaction medium, were studied. The nanocapsules were evaluated for the ability to protect the essential oil from thermal processes similar to those applied in polymer film and paper film processing.

1.1 **Aims of this study**

The aim of this thesis is to synthesize the geranyl cinamate from the lipase catalyzed enzymatic esterification of geraniol and cinnamic acid and to protect this compound through its nanoencapsulation with polycaprolactone polymer.

In particular, the specific objectives are:

- ✓ To define an enzymatic route to obtain the geranyl cinnamate ester;

- ✓ To evaluate the antibacterial and antifungal activity of the geranyl cinnamate ester in the presence of common pathogenic bacteria and fungi in food;
- ✓ To define a nanoencapsulation route of the geranyl cinnamate ester with polycaprolactone polymer;
- ✓ To characterize the nanocapsules obtained in terms of average particle size, polydispersity index, zeta potential and encapsulation efficiency, as well as their morphological characteristics.
- ✓ To evaluate the stability of the nanocapsules of polycaprolactone and geranyl cinnamate ester in suspension over the storage time of 60 days;
- ✓ To increase the thermal stability of the geranyl cinnamate ester with the protection of nanoencapsulation with polycaprolactone.

1.2 Structure of this document

This thesis is organized as follows:

In Chapter 2 a review of recent studies related to nanotechnology and nano and microencapsulation of essential oils is presented. This study provides valuable insight that may be useful for identifying trends in the commercialization of nanotechnology products or for identifying new research areas. This chapter is based on the paper entitled "**Use Of Encapsulated Natural Compounds as Antimicrobial Additives in Food Packaging: A Brief Review**" (Trends in Food Science & Technology - the article is under review after the second correction.- Ref: TIFS_2017_242_R2), by Micheli Zanetti, Thaís Karoline Carniel, Francieli Dalcanton, Raul Silva dos Anjos, Humberto Gracher Riella, Pedro H. H. de Araújo, Débora de Oliveira, Marcio Antônio Fiori. In summary, the review presents discussions on the use of synthetic chemical preservatives in food, which can be harmful to human health. These problems have increasingly aroused the concern and interest of researchers, leading to the study and application of non-toxic essential oils with preservative capacity in food products and food packaging. A major challenge in this regard is its easy degradation during processing of food and manufacturing processes during food packaging. The encapsulation is presented as an interesting technique to improve the physical-chemical and microbiological stability of these essential oils, as well as to obtain controlled release. This review provides a detailed overview of encapsulation in the food industry, focusing on the

application of procedures to encapsulate essential antimicrobial oils. The results published to date confirm that the use of nanoencapsulation promotes the protection of active compounds, enabling industrial applications of active packaging.

In Chapter 3, the ability of pure geraniol and cinnamic acid to act as antimicrobial agents against 4 different bacteria is presented. This chapter is based on the paper entitled "**Microbiological Characterization of Pure Geraniol and Comparison with Bactericidal Activity of the Cinnamic Acid in Gram-Positive and Gram-Negative Bacteria**" by Micheli Zanetti, Raquel Z Ternus, Francieli Dalcanton, Josiane M.M. Mello, Humberto Gracher Riella, Pedro H. H. de Araújo, Débora de Oliveira, Marcio Antônio Fiori, published in Journal of Microbial & Biochemical Technology (Volume 7(4): 186-193, 2015). Geraniol is a terpene alcohol and the principal constituent compound of many essential oils of the aromatic plants. This molecule is very important for the flavors and fragrance industries due to its pleasant odor. The geraniol has the insecticidal properties and is an efficiently repellent agent and its toxicity is low. So, its antimicrobial ability can be explored to obtaining important additives for especial products applied in the food industry. The cinnamic acid is a molecule present in cinnamon oils and in coca leaves. This molecule also exhibits low toxicity and has a broad biological application spectrum for many microorganisms. This work evaluated the antimicrobial activity of the essential oil of geraniol and of the cinnamic acid against different microorganisms and compared their bactericidal activity for future applications as an additive for food packages. The bacteria studied were: *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Salmonella enterica*.

In Chapter 4, the route of obtaining the geranyl cinnamate ester from the geraniol essential oil and the cinnamic acid is presented. This chapter is based on the paper entitled "**Synthesis of Geranyl Cinnamate by Lipase-Catalyzed Reaction and its Evaluation as an Antimicrobial Agent**", by Micheli Zanetti, Thaís Karoline Carniel, Alexsandra Valério, J Vladimir de Oliveira, Débora de Oliveira, Pedro H.H. de Araújo, Humberto Gracher Riella and Marcio A. Fiori, published in Journal of Chemical Technology and Biotechnology (v. 92, p. 115-121, 2017). To be as generic as possible, through experimental design 75% of geranyl cinnamate was obtained under reaction conditions of 70 °C, 15 wt% of immobilized *C. antarctica* NS88011, and 3:1 geraniol to cinnamic acid molar ratio. Kinetic assay showed it was possible to improve the enzymatic esterification reaction (97%) under reaction conditions of 80

°C, 20 wt% of immobilized NS88011 lipase and 5:1 geraniol to cinnamic acid molar ratio. The antimicrobial activity of the geranyl cinnamate ester was tested on bacteria *Staphylococcus aureus* and *Escherichia coli* by determining the minimum inhibitory concentration (MIC) and agar diffusion. The compound showed excellent antimicrobial activity for the two bacteria, with a bactericidal effect. The results showed the promise of a technique to overcome thewell-known drawbacks of the chemical-catalyzed route to obtain a high-value compound.

In Chapter 5, is presented the nanoencapsulation process, as well as the characterization of the nanocapsules obtained. This chapter is based on the paper entitled "**Encapsulation of geranyl cinnamate in polycaprolactone nanoparticles"**, by Micheli Zanetti, Alessandra Cristina de Meneses, Luciano Luiz Silva, Pedro Henrique Hermes de Araújo, Márcio Antônio Fiori and Débora de Oliveira, submitted in Materials Science and Engineering: C. In this work we investigated the encapsulation of geranyl cinamate in polycaprolactone (PCL) and the antibacterial properties of nanocapsules against Escherichia coli and Staphylococcus aureus. The results show that nanocapsules with an average size of 177.6 nm and a polydispersity index of less than 0.200 were obtained, showing the formation of nanocapsules with a homogeneous size. Electron microscopy results showed the formation of spherical nanocapsules and the TGA results showed the thermal protection of the ester by the PCL coating. The encapsulation increased the temperature for the release of the geranyl cinnamate ester at 20 ºC. Suspensions of geranyl cinnamate ester nanocapsules stored at 4 ºC showed good stability over 60 days. Minimum inhibitory concentration (MIC) tests showed that the geranyl cinnamate compound is not released by the PCL nanocapsules up to 72 hours time in solution, requiring the use of a trigger (temperature e.g) to disruption of the capsule and release of the active compound.

Chapter 6 presents tests in progress, with pre-liminary results that have not yet been published, and these may serve as a basis for future work. This chapter was designated as supplementary material and was divided in 3 items. In item 1 is presented the nanoencapsulation of geranil cinnamate by polycaprolactone as a thermal protection method. In item 2 the ability of the geranyl cinnamate ester to act as an antifungal agent for the fungi *Aspergillus niger*, *Penicillium variabile* and the yeast *Candida albanis* is presented. And in section 3 tests are presented to verify the toxicity of the geranyl cinnamate ester. The initial test was performed via the acute systemic toxicity test, using a concentration of 300 mg of compound for each kg of animal.

Chapter 7 presents the essence of this document, i.e., a critical summary of what was discussed in chapters 2-5 and what to expect in the future. It provides a concise link between all ideas discussed herein, aiming to provide to the reader the big picture of the investigation carried out and the real meaning of the contributions proposed in this thesis.

Finally, in Chapter 8 all references used in this document are listed.

2 USE OF ENCAPSULATED NATURAL COMPOUNDS AS ANTIMICROBIAL ADDITIVES IN FOOD PACKAGING: A BRIEF REVIEW

This chapter is based on the paper entitled "USE OF ENCAPSULATED NATURAL COMPOUNDS AS ANTIMICROBIAL ADDITIVES IN FOOD PACKAGING: A BRIEF REVIEW" (Trends in Food Science & Technology - the article is under review after the second correction.- Ref: TIFS_2017_242_R2), by Micheli Zanetti, Thaís Karoline Carniel, Francieli Dalcanton, Raul Silva dos Anjos, Humberto Gracher Riella, Pedro H. H. de Araújo, Débora de Oliveira, Marcio Antônio Fiori. Abstract, keyworkds and acknowledgements were omitted.

2.1 Introduction

The encapsulation of active compounds has appeared relatively recently, but its rapid and significant advances have allowed its use in the most diverse branches of the industry, mainly pharmaceutical, cosmetic and food sectors.

Encapsulation is a process which entraps one substance (active agent) into another substance, called wall material, producing particles in the nanometer (nanoencapsulation), micrometer (microencapsulation) or millimeter scale (Burgain et al., 2011), by many encapsulation techniques. There are many proposed encapsulation procedures, but none of them can be considered as a universal applicable procedure.

Nano/microencapsulation technology of these nanostructures can be a practical and efficient approach to solve problems such as the physical instability and bioactivity of some important active molecules and sometimes to control the release of active molecules to the external medium around the nanocapsules by diffusion processes (Aloui et al., 2014). Nanocapsules are typically obtained in powder form and exhibit properties that are different from larger powder particles with the same chemical composition (Sekhon, 2010).

Nanocapsules are unique structures composed of a polymeric shell deposited around a core. The active component of interest in the core is dissolved to the polymeric wall. It is interesting to note that nanocapsules are very small, thousands of times smaller than a bacterium (Assis et al., 2012).

Encapsulation favors the delivery of the active compounds of interest, as it may be allocated in a suitable carrier delivering a targeted delivery, and still has a release controlled by different time intervals

independent of the mass molecular or bioactive nature, improving safety and effectiveness since it benefits the protection of the bioactive compound.

The rapid advance in nanotechnology in recent decades has boosted the development of advanced techniques to release active compounds in diverse processes, especially in biological mechanisms (Donsi et al., 2011). The production of polymeric nanocapsules has been a focus in the pharmaceutical and food industries, as well as the scientific community, as polymeric nanocapsules have interesting characteristics and properties for use as sustainable release systems of active compounds (Brandelli and Taylor, 2015).

Wrona et al. (2017) showed that the encapsulation was successfully used for the preparation of active materials, where tea extract was incorporated into polyethylene by extrusion technology. It was demonstrated that inorganic capsules protected the extract of green tea versus processing temperature by extrusion. The release of a small portion of active agent at parts per billion (ppb) level from the active material was confirmed. Then, effectiveness of the developed packaging was checked with in vivo experiments and the extension of shelf-life of fresh minced pork meat was successfully achieved.

However, for their application in scaled and industrial processes, it is necessary to consider the limitations of essential oils, such as their low solubility in water, organoleptic taste and especially low thermal stability. The encapsulation of essential oils appears to be an important alternative to overcome such limitations. Coating of these natural compounds with more resistant nanostructured materials increases the thermal and chemical stability, as well as the solubility in solvents under certain conditions. The encapsulation of essential antimicrobial oils also benefits controlled release, increases the cellular bioavailability and increases the efficiency against pathogens (Rai et al., 2017).

Over time, the food industry has undergone constant changes to adapt to the growing demands of food safety. The demand for minimally processed products, such as natural foods, requires certain properties and special characteristics of the packaging, mainly pertaining to the shelf life of the conditioned products.

Based on these demands, several new technologies and packaging materials have recently been investigated and developed, such as active packaging, smart packaging, edible coatings/films, biodegradable packaging, etc. (Ghaani et al., 2016; Kapetanakou and Skandamis, 2016).

Active packaging is defined as having some role in food preservation other than forming an inert barrier to external influences

(Azeredo et al., 2000). Active packaging is an innovative approach to maintain or prolong the shelf- life of food products while ensuring their quality, safety, and integrity. As defined in the European regulation (EC) No 450/2009, active packaging comprises packaging systems that interact with the food in such a way as to "deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food" (European Commission 2009).

The unique properties of active packaging are obtained from the direct incorporation of compounds with specific properties, for example, antimicrobial compounds. These compounds, when properly studied, exert the function of special additives and can be incorporated during the manufacturing process of the packaging.

Antimicrobial essential oils provide a great opportunity for the development of these additives, since they are generally non-toxic and because they have important properties for food preservation. Some essential oils are antimicrobial and antioxidant and, when protected by nanocapsules coatings from degradation effects of the processing equipment, can be used to produce active antimicrobial or high-performance antioxidant packaging.

In this review, we present recent research related to encapsulation of essential oils in the manufacture of active food packaging. This study provides valuable insight that may be useful for identifying trends in the commercialization of nanotechnology products or for identifying new research areas.

2.1 **The use of nanotechnology for the development of antimicrobial packaging**

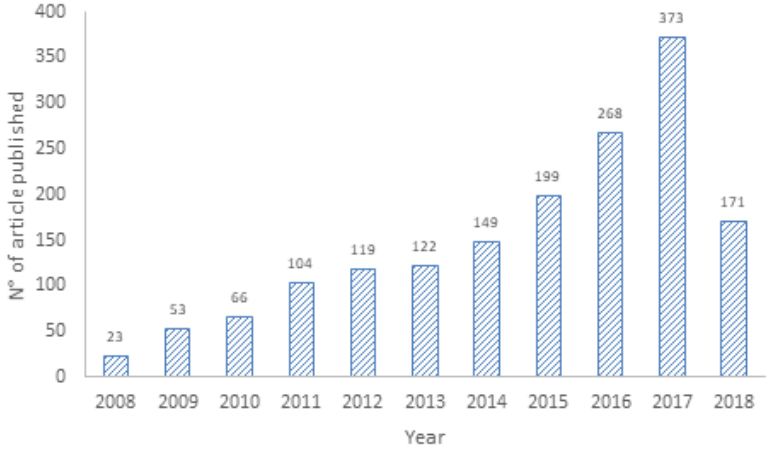
The manipulation of matter at the nanometer scale represents an emerging discipline (nanoscience and nanotechnology) and has become an important topic in the food industry. The application of nanotechnology to protect foods is recent compared to the use of this technology in the biomedical and electronic industries, in which nanotechnology is already used in the manufacture of materials and devices. However, there are already numerous opportunities that can be exploited, such as the development of products with functional and nutraceutical characteristics, process development and smart packaging using particular nanoparticles as food additives, for example (Assis et al., 2012).

The traditional synthetic chemical preservatives used by the food industries no longer meet the demands of consumers that seek not only

safe but also more nutritious and tasty foods. Thus, the use of antibacterial and antifungal nanoparticles to obtain antimicrobial food packaging and the use of antioxidant nanoparticles to preserve foods have been a large study field by the food industries.

Figure 2.1 shows the evolution of the amount of publications related to nanotechnology processes applied to food packaging in the last 10 years (Source: ScienceDirect - Elsevier). It is possible to observe a significant growth in the amount of scientific publications in the last decade concerning this subject. The number of studies associated with food packaging, food additives and food supplements has also been investigated (Mozafari et al., 2008).

Figure 2.1- Number of publication related the use of nanotechnology for the development of antimicrobial packaging in the last 10 years.



Fonte: http: www.sciencedirect.com (2018)

Practically all recent studies in this subject have been explored the improvements in the properties that the nanoparticles manifest. The processes with nanoscale control can be used to modify the structure of food additives and to improve the quality of foodstuffs, as well the taste, flavor, color, texture, and consistency (Mozafari et al., 2008). These technologies applying nanoparticles as food additives are used currently by more than 400 companies around the world, including Kraft, Nestle, Unilever, Keystone, Heinz, Hershey, etc.

Nanoencapsulation process, for example, is an interesting and promising method applied by the food industries to obtain special antimicrobial and antioxidant nanoparticles with the protection function of the foods (Katouzian and Jafari, 2016). The encapsulation of bioactive substances protects its the active molecules from degradation processes and to improve their physical and chemical stability and solubility in foods (e.g., solubilization of hydrophilic components and hydrophobic matrices and vice versa) (Jafari et al., 2008).

With the development of active packaging, the nanotechnology has great application potential by the food industry, as it can provide alternative functions, such as the development of special nanoparticles to improve the nanodispersion of food additives or the production of nanolaminates and nanotubes materials, which when associated with certain polymers, can provide several food protection functionalities. For example, some research in the area of food packaging, has indicated that the properties of many materials can be improved with the addition of nanocomposites during its development (Espitia et al., 2012), or the incorporation of silver nanoparticles (Metak, 2015) or titanium dioxide (TiO2) nanocomposites with antimicrobial properties (Lahtinen et al., 2013) as well as the addition of silicate nanocomposites to improve the properties, reducing the hydrophilicity.

Food packaging materials comprise the largest category of applications of nanotechnology in the food sector, in special for the use of particular nanoparticles. Several authors have discussed the main developments in packaging derived from nanotechnology, which are presented in Table 2.1.

Table 2.1 Some studies from the literature about the application of nanotechnology in food packaging.

Application	Reference
Incorporation of nanomaterials that improve packaging properties (flexibility, gas barrier properties, temperature / humidity stability)	Gatos, 2016
Incorporation of metal oxide or metal nanoparticles in packages (e.g. silver, zinc oxide, magnesium oxide) with antimicrobial properties	Al-Naamania et al., 2016
Intelligent food packaging, incorporating nanosensors to monitor and report food conditions	Nagarajan et al., 2017
Nanocoatings with antimicrobial or barrier properties and for self-cleaning surfaces in food processing facilities.	Brandelli and Taylor, 2015
Nanoencapsulation of bioactive compounds to control environmental conditions in food packaging	Fathi et al., 2012

Despite its immense potential, antimicrobial food packaging is little used, to date, on a large scale. Currently used antimicrobial agents are oxidizing ions, such as copper or silver ions, that present limited efficacy against microorganisms, low thermal stability and mostly function through direct contact (Brandelli, 2015; Realini and Marcos, 2014). Most of the antimicrobial packages are produced using as antimicrobial compounds silver nanoparticles or ionic doped glass microparticles as antimicrobial compounds (Mendes et al., 2012; Thomé et al., 2012; Seydim et al., 2006; Fiori et al., 2009a; Paula et al., 2009; Santos et al., 2011; Fiori et al, 2009b). These traditional additives are generally toxic at low concentrations and their use in antimicrobial packaging can cause great concern for consumers. Thus, the interest of application of the antimicrobial and antioxidant compounds that present the thermal stability and high antimicrobial activity has been a great expectation of the scientific studies and industrial studies.

In this context, the use of natural extracts and essential oils as non-toxic additives for antimicrobial packaging is a great opportunity to minimize these problems. Many interesting studies have been investigated the application of natural extracts and essential oils encapsulated in nanoparticles as additives to produce food packaging, for example. The possibility of incorporating these natural antimicrobial compounds directly into the polymer films or into cellulose provides good possibilities for the production of non-toxic antimicrobial food packaging and the nanoencapsulation with polymeric material have been a good strategy for the thermal protection. These antimicrobial packs have been tested to inhibit the growth of important pathogenic microorganisms and to protect the packed food.

2.2 **Natural extracts and their application in active packaging**

The main purpose of food treatments is to provide well-being to the consumer through safe, nutritionally adequate food while meeting the expectation of taste, aroma, appearance and comfort. Furthermore, the desire of modern society to consume fresh food has increased the demand for minimally or partially processed food.

Natural plant extracts can assist in maintaining the appearance, taste and quality of food without any negative impact on the color, odor and taste profiles. A large variety of extracts have effective antioxidant and antimicrobial properties, constituting alternatives to conventional synthetic preservatives.

Although most of the preservatives used in food are of synthetic origin, there are several products from naturally occurring plants that can be used as food preservatives. It is estimated that 1 to 10% of the 500,000 species of plants in the world, that is, approximately 500, have use as food preservatives (Sauceda, 2011).

Traditional methods of preservation, such as freezing, pasteurization, sterilization and dehydration, are based on the manipulation of one or two conservation factors. Currently, combinations of two or more factors that interact in additive or synergistic ways to control microbial populations are sought, avoiding the application of a single severe conservation factor, which improves the sensorial and nutritional quality of the food, producing fresh, healthy products with fewer additives that are ready to prepare and serve (Alzamora, 1997).

The first studies on the development of active packaging are related to the use of inorganic compounds, for example, silver (Ag)

nanoparticles have been widely employed as food packaging additives to increase the shelf life of food by controlling the growth of pathogens. Metak (2015) tested Ag nanoparticles in polyethylene packages used for fresh apples, sliced white bread, fresh carrots, cheeses, and fresh orange juice. The nanoparticles were efficient at controlling the bacteria *Staphylococcus aureus*, *Escherichia coli* and *Listeria*. Positive effects, such as the preservation of appearance and sensory quality, were obtained when storing strawberries in low-density polyethylene packages incorporated with Ag nanoparticles (Yang et al., 2010).

Cooper nanoparticles are considered an alternative to replace the antimicrobial activity of Ag nanoparticles (Bergin and Witzmann, 2013). The antimicrobial activity of Cu nanoparticles has been proven to be effective against Gram-positive and Gram-negative bacteria and yeast (Usman et al., 2013).

However, some studies have shown that silver nanoparticles are toxic to humans (Carbone et al., 2016), as well as copper nanoparticles (Hannon et al., 2016). In 2014, the US FDA published a document that provides guidance to manufacturers of food ingredients and food contact substances. The US FDA recommends that manufacturers study and prepare a toxicological profile for each container incorporated with nanomaterials (USFDA, 2014). In March of 2014, the United States Environmental Protection Agency (EPA) prohibited the sale of plastic food containers with nanosilver produced by an American company because their products have not been tested according to US FDA regulations (Martin, 2014).

Then some studies have shown the combination of inorganic compounds such as silver, gold, zinc, chitosan, platinum, iron, copper, carbon nanotubes and many others, have been used with essential oils to evaluate their antimicrobial activity (Gaspar et al., 2017; Jogee et al., 2017; Van Long et al., 2016). Different types of nanocapsules are used for the preparation of nanocomplexes, which enhance the bactericidal and fungicidal activity of essential oils (Gomes et al., 2011). At the same time, concern about the application of inorganic nanoparticles has increased due to the toxicity (García et al., 2011) of some types of nanoparticles, and for this reason, scientists have moved towards the fabrication of non-toxic, biocompatible and biodegradable nanoparticles (Pinto et al., 2016).

Because they are non-toxic and naturally occurring compounds, the ability of various essential oils to act as food preservatives due to their antimicrobial properties has been studied by many researchers (Alwan et al., 2016). Studies evaluating the efficacy of combating pathogens in different applications (pharmaceutical, cosmetic, etc.), mainly in food and

packaging, have shown essential oils to be a promising scientific and technological approach (Knezevic et al., 2016).

The most active antimicrobial compounds present in essential oils can be divided into four groups based on the chemical structure: terpens (e.g., p-cymene, limonene), terpenoids (e.g., thymol, carvacrol), phenylpropenes (e.g., eugenol, vanillin) and other compounds such as allicin or isothiocyanates (Hyldgaard et al., 2012). The mechanism of action of essential oils against microorganisms has not yet been elucidated entirely and cannot be attributed to a single mechanism. There are several locations in the microorganisms which are supposed to be the sites of the action for EOs (Nazzaro, et al., 2013).

The antimicrobial activity of essential oils, similar to all-natural extracts, is dependent on their chemical composition and number of components. Many antimicrobial compounds are constitutively expressed by plants, and others can be synthesized by self-defense mechanisms in response to pathogens (Nazzaro et al., 2013). Essential oils contain a wide range of secondary metabolites that can inhibit or retard the growth of bacteria, yeasts and molds (De Martino et al., 2009).

Essential oils and their components have a variety of targets, particularly the membrane and cytoplasm, and in certain situations, they completely alter the cell morphology (Nazzaro et al., 2013). Generally, Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria (Trombetta et al., 2005). Geraniol essential oil is more active towards *Staphylococcus aureus* (Gram-positive) than towards *Escherichia coli* (Gram-negative) (Zanetti et al., 2015).

López et al. (2005) studied the antimicrobial activity of essential oils of cinnamon (*Cinnamon zeylanicum*), clove (*Syzygium aromaticum*), basil (*Ocimum basillicum*), rosemary (*Rosmarinus officinalis*), dill (*Anethum graveolens*), and ginger (*Zingiber officinalis*) over a range of concentrations in two types of contact tests (solid and vapor diffusion). The essential oils were tested against an array of four Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, and *Listeria monocytogenes*), four Gram-negative bacteria (*Escherichia coli*, *Yersinia enterocolitica*, *Salmonella choleraesuis*, and *Pseudomonas aeruginosa*), and three fungi (a yeast, *Candida albicans*, and two molds, *Penicillium islandicum* and *Aspergillus flavus*). The fungi were the most sensitive microorganisms, followed by the Gram-positive bacterial strains. The Gram-negative strain *P. aeruginosa* was the least inhibited.

The effects of cinnamon and mustard essential oils against nine foodborne bacteria were evaluated by Clemente et al. (2016). Their antimicrobial susceptibility and action mechanism was also assessed.

Additionally, the effects of the combination between both were evaluated. The results indicated that the mustard presents 10 times more bactericidal/bacteriostatic effect than cinnamon essential oil.

Cinnamon essential oil has been used for centuries to protect food from microbiological infection, and in the last ten years' cinnamon essential oil is also incorporated into food packaging materials as antimicrobial agent. Manso et al. (2013) tested polyethylene terephthalate films containing cinnamon essential oil in vapor phase, without direct contact with the mold. The combined results demonstrate the effectiveness of cinnamon essential oil as antifungal compound, independently of the substrate from which it is delivered.

In the study carried out by Gherardi et al. (2016) an innovative procedure to incorporate an antimicrobial agent in a multilayer active material was used to prepare several antimicrobial packaging materials. The active materials were prepared containing polyurethane adhesive free of isocyanates and different concentrations of cinnamon essential oil as active agent. The antimicrobial activity of the material was evaluated in tomato puree, obtaining high efficiency for *Escherichia coli* O_{157}:H_7 and *Saccharomyces cerevisiae*.

Essential oil components such as carvacrol and cinnamaldehyde have been incorporated, for example, in apple edible films with proved anti-*Campylobacter jejuni* activity, being able to yield bactericidal action against *C. jejuni* cells inoculated on chicken breasts, with cinnamaldehyde exerting a higher antimicrobial activity (Mild et al., 2011).

The antimicrobial properties of Zataria multiflora, Iranian and European Cumin were evaluated by Akrami et al. (2015), first as pure extracts and later as active agents incorporated in paper, manufactured at laboratory scale as an active packaging material. All bacteria tested (*Staphylococcus aureus*, *Listeria innocua*, *Pseudomonas sp.*, *Salmonella enterica sp*. and *Escherichia coli*) were inhibited when exposed to the atmosphere generated from 4 to 6% (w/w) of Zataria essential oil in the active coating.

De Souza et al. (2016) measured the inhibitory effects of *Origanum vulgare L.* (0.6, 1.25, 2.5 or 5 µL/mL) on *S. aureus*, *L. monocytogenes* and *L. lactis* ssp. cremoris in "coalho" cheese broth and slurry. MIC of essential oil against *S. aureus* and *L. monocytogenes* were 2.5 µL/mL and 0.6 µL/mL for mesophilic lactic acid bacteria. During 24 h, 0.6 µL/mL essential oil had no inhibitory effect toward both pathogenic bacteria in cheese broth whilst viable cell counts of starter culture decreased 1 log CFU/mL.

In a work performed by Zantar et al. (2014), essential oils of *Thymus vulgaris* and *Origanum compactum* (0.05 and 0.1%) were added to goat cheese and their effects on some pathogenic and spoilage microorganisms were studied. Assessing antibacterial activity by disk diffusion method showed that the highest antibacterial activity for *T. vulgari* against *S. aureus*.

The antifungal activity of essential oils is also cited. In the study of Frias and Kozusny-Andreani (2008), were extracted essential oil of lemon and citronella, as well as some components of the essential oils citral, geraniol, citronellol and citronellal, were tested in four pathogenic fungi (*Candida albicans*, *Microsporum gypseum*, *Sporothrix schenckii* and *Aspergillus niger*), and lemon oil showed high antifungal activity for all tested organisms. The same authors verified in their study that the essential oil of eucalyptus had a high antifungal activity against *Aspergillus niger*.

Recently, it has been demonstrated that the use of an active packaging based on the incorporation of essential oils caused inhibition or reduction in the production of mycotoxins, both in direct contact and in the vapor phase. Clemente, Aznar and Nerín (2016) showed that the incorporation of benzyl isothiocyanate into packs caused changes in essential cellular components, such as saccharides, amino acids, proteins, lipids or enzymes, impacting various cellular functions such as respiration, metabolism or cell cycle. All these metabolic changes will affect the production of *Aspergillus ochraceus*, which makes benzyl isothiocyanate a great antifungal agent for use in packaging to ensure food safety.

However, a serious problem in the incorporation of essential oils in polymer matrices is that if pure oil is added during the manufacture of conventional packaging, it will be volatilized and degraded during the process, generating significant mass loss and compromising the antimicrobial function of the polymers. The main causes of decomposition are the high shear rates and high temperatures applied during processing in the manufacture of packaging (Malhotra et al., 2015).

To protect essential oils, which are very volatile and can suffer from thermal degradation and photodegradation, nanoencapsulation is an important procedure. It is a technique in which solid, liquid and gaseous substances, compounds or agents are coated with an encapsulating agent, thereby obtaining nanometer-sized particulates. Some studies have shown that these compounds can be protected via encapsulation using different

encapsulating compounds, such as natural mineral nanotubes and aluminosilicate clay (Lvov et al., 2015).

In studies carried out by Ribeiro-Santos et al. (2016), mixtures of essential oils of oregano, cinnamon and sweet fennel were incorporated into cellulose acetate films to thus obtain films with high antimicrobial activity against *Penicillium spp*. and *Staphylococcus aureus*. Gomes et al. (2011) studied the incorporation of eugenol and cinnamaldehyde encapsulated with poly(lactic acid-co-glycolic acid) (PLGA), which proved to be a potential bactericidal agent against *Salmonella* and *Listeria*.

Beyki et al. (2014) achieved improved antimicrobial activity against *Aspergillus flavus* using Mentha essential oil encapsulated in chitosan/cinnamic acid nanogels. When applied in food packaging, Wen et al. (2016), demonstrated that polyvinyl alcohol and cinnamon essential oil in β-cyclodextrin (PVA/CEO/β-CD) form an antimicrobial film that can effectively extend the shelf life of strawberries, indicating their potential for application in active food packaging.

According to Becerril et al. (2007), the design and production of a new antimicrobial material is not an easy task. Once the active agents have been introduced into the matrix it is necessary to study its behavior. Some techniques should be used to verify the effectiveness of the oil incorporated: a) specific microbiological tests to check the activity of constituent antimicrobial agents and to determine if the direct contact between the antimicrobial agents is required or if a small amount of them in vapor phase is enough to achieve total inhibition of microorganism growth; b) the kill time test to establish the lag time required by each microorganism to be altered by the active agent; c) chromatographic analysis, usually gas chromatography-mass spectrometry (GC-MS) to identify and quantify the individual compounds released by the packaging material; d) usually a micro-extraction technique is applied to extract the compound in the headspace vapor in equilibrium with the packaging material (López et al., 2006); e) electronic microscopy to investigate the microorganism cell damage produced by the active compounds; f) and finally a method to determine which individual compound, or mixture of compounds, penetrate(s) through the cell wall and accumulate in it, thereby affecting the cell survival or its growth.

Barros-Velázquez (2016) say that active food packaging strategies have experienced a tremendous push forward in the last two decades. During this time, the development of newer materials and the incorporation of bioactive compounds in the packaging films have represented a revolution in the food industry and have run in parallel (and

sometimes spurred by) with the development of advanced techniques for the detection of emerging and resistant food-borne pathogens.

2.3 Encapsulation as protection of Natural Extracts

Studies have shown that active packaging not only forms an inert barrier to external influences but also interacts with the product to demonstrate important properties for food protection and consumer safety. Table 2.2 presents a summary of studies that evaluate the application of active compounds in the food industry.

In turn, nanocapsules are structures formed by a coating, usually polymeric, arranged around a core containing an active component that is dissolved and/or adsorbed to the wall of the coating. Nanoparticles have sizes smaller than 100 nm, and therefore, new phenomena occur because of these characteristics, which afford new applications and benefits (Sekhon, 2010).

Table 2.2 – Application of active compounds in different sectors of food industries.

Active compounds	Biological activity	Food product	Reference
Lemon and citronella	Antifungal	Food deteriorated by fungi	Frias and Kozusny-Andreani, 2008
Essential oils of eucalyptus	Inhibition of growth and color change in fungal mycelia	Food deteriorated by fungi	Salgado et al., 2003
Eugenol, thymol and carvacrol	Antimicrobial	Food deteriorated by bacteria	Castillo et al., 2014
Cinnamon leaf essential oil	Increased total phenolics and antioxidant capacity; improved odor acceptability	Fresh-cut peaches	Ayala-Zavala et al., 2008
Thyme, cinnamon leaf, and garlic essential oils	Antifungal	Fruits and vegetables	Ayala-Zavala et al., 2008
Clove, rosemary, lemon, melisa, and tea tree essential oils	Reduced peroxidase activity	Swiss chard, spinach, butter lettuce and cabbage	Ponce et al., 2004

The nanoencapsulation of essential oils, for example, can be a practical and efficient approach to solve problems such as the physical instability of these compounds and to increase their bioactivity. As an example, nanoencapsulation can be used for the post-harvest control of fruits by reducing the diffusion and maintenance of high concentrations of active molecules on the fruit surface (Aloui et al., 2014).

Schmitt et al. (2016) prepared a super-lattice of essential oil droplets in an emulsion with gold NPs, whereas Duncan et al. (2015) synthesized capsules with peppermint essential oil and cinnamaldehyde in the core, stabilized by NP encapsulation. A similar study using different techniques was reported by Paula et al., (2016) who prepared chitosan-gum NPs loaded with thymol, containing the essential oil of *Lippia sidoides*. This nanoencapsulation was achieved by spray-drying.

The encapsulation of eugenol, thymol and carvacrol in nanometric surfactant micelles also resulted in improved antimicrobial activity (Chen et al., 2015). Nanoencapsulation has been observed to shows signs of more widespread use, but its application in industrial products is still in the initial stages.

Other essential oils, like chia, were encapsulated with whey protein concentrate (WPC) with mesquite gum and WPC + Arabic gum, using spray dry (Rodea-Gonza´ lez et al., 2016) Summer savory essential oil loaded chitosan nanoparticles (NPs) were produced. The particle size of the NPs ranged from 140.25 to 237.60 nm at different pH levels and encapsulation efficiency was in the range of 40%, the NPs showed strong antibacterial and antioxidant activities against *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* O_{157}:H_7 (Feyzioglu and Tornuk, 2016).

Much effort has been directed towards the incorporation of these sensitive compounds into commodity polymers (polyethylene, polypropylene, polystyrene, and poly(vinyl chloride)) by conventional manufacturing techniques while minimizing their loss during high-temperature processing and maintaining their antimicrobial function (Malhotra et al., 2015). Recently, the use of Halloysite nanotubes (HNTs), which are natural aluminosilicate clay minerals (Lvov et al., 2015), as nanoscale carriers for carvacrol, used as a model essential oil, was demonstrated. The entrapment of carvacrol within HNTs allows the incorporation of this highly volatile essential oil into different polymer matrices at elevated temperatures of up to 250 °C (Shemesh et al., 2016).

Silva et al. (2015) investigated the anti-*Campylobacter* activity and mode of action of stilbenes encapsulated in cyclodextrins. Results showed that pterostilbene and pinosylvin and their inclusion complexes (ICs) had antimicrobial activity against *Campylobacter jejuni* and *Campylobacter coli*. Furthermore, pinosylvin ICs had a bactericidal activity against both *Campylobacter* species at 37 °C and even at 4 or 20 °C. This bactericidal action is due to membrane damage, resulting in the impairment of several cellular functions such as membrane polarization, permeability and efflux activity.

Several materials used in food packaging can be used to incorporate essential oils. Applicable polymeric matrices can be derived from non-renewable materials, such as polypropylene, or from biomaterials, such as chitosan (Azeredo et al., 2010). The use of aromatic plants has afforded a large variety of essential oils from different aromatic sources and has been applied to food packaging, being Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) (FDA, 2016). One of the main trends in the area of food packaging is the use of biodegradable materials from renewable sources, which contribute to food chain sustainability (Ribeiro-Santos et al., 2017).

One major obstacle in the use of essential oils is the lack of reproducibility of their activity. Despite the great diversity of compounds, they contain, essential oils may have qualitative and quantitative variations in the content of bioactive components, resulting in variable biological effectiveness (Li et al., 2013). Moreover, another limitation to the use of essential oils in foods is due to their strong aroma, which may restrict their applications.

Several encapsulation systems, with different formulations and size distributions were tested for the delivery of essential oils in foods. For example, the use of a solid-fat micrometric emulsion was reported to reduce the volatility of the antimicrobial agent, entrapped in the crystallized fat structure, thus protecting the active compounds against environmental factors (Gavini et al., 2005).

In the literature, it is possible to find several preparation methods for polymer nanoparticles, each with advantages and disadvantages. These methods can be divided into two main categories: those whose preparation requires a monomer polymerization reaction, such as emulsion polymerization, miniemulsion and interfacial polymerization reactions, and those that use pre-formed polymers, such as emulsification with solvent evaporation, nanoprecipitation, solvent diffusion and salting-out.

A full investigation of the main techniques for producing nanocapsules used in the food industry was carried out. The compiled information is presented in Table 2.3.

Table 2.3 - Techniques of nanoencapsulated of compounds used and application in food industry processes.

Encapsulated compound	Technique of nanoencapsulation	Application	References
Nano-encapsulation of fish oil in nano-liposomes	Emulsification by solvent evaporation	Fortification of yogurt	Ghorbanzade et al., 2016
Nano-encapsulation of vitamins	Emulsion technique	Bioavailability of vitamin E in fortified breakfast cereal	Leonard et al., 2002
Vitamin D2	Coacervation	Effect of process variables on the encapsulation process	Junyaprasert et al., 2001
Thymol, carvacrol, limonene, and Cinnamaldehyde	Nanodispersion	Enhanced antimicrobial activity	Shah et al., 2012
Eucalyptus oil	Nanoemulsion	Enhanced antibacterial activity	Saranya et al., 2012
Lemon myrtle oil	Nanoemulsion	Enhanced antimicrobial properties	Buranasuksombat et al., 2011
Polyamine membranes and cyanuric chloride	Interfacial polymerization	Film composed of polyamine membrane	Lee et al., 2015
Natural phytochemicals and probiotics	Spray drying Freeze drying Coacervation Yeast-encapsulation	Functional food	Silva et al., 2016
β-carotene	Nanoemulsification and supercritical fluids	Natural pigments - component of the food industry	Gutiérrez et al., 2013

The choice of nanocapsules preparation technique depends on the characteristics of the bioactive compound, such as hydrophilicity or lipophilicity, solubility, and stability, and the desired properties of the product, such as the particle size and bioavailability, among others.

The advantages and limitations of its use in the food industry have not been fully elucidated to date. The major challenges are to gather concise information on the properties and risks of these nanomaterials in applications on an industrial scale. However, all studies aimed at solving these doubts are fundamental, as they will allow the development of better quality products with greater security and lower cost.

2.4 **Potential applications of nanocapsules with essential oil in food packaging**

Active packaging passively protects food items, not only inhibiting pathogenic growth but also providing extended shelf life by combating a variety of environmental factors. Since antimicrobial agents have a higher surface/volume ratio compared to their normal counterparts, they are considerably more efficient against foodborne pathogens (de Azeredo, 2013).

Food safety can be ensured through the use of antimicrobial coatings in food packaging (Makwana et al., 2014). The use of active natural extracts in food preservation is an alternative to chemical preservatives and assists in satisfying consumer demand for nutritious and safe products, free of synthetic additives.

When applied to food, essential oils can perform antimicrobial, antioxidant or flavoring functions. The most important action of an essential oil is to minimize or even eliminate the presence of microorganisms and/or reduce the phenomenon of lipid oxidation (Ribeiro–Santos et al., 2017). These essential oils can be added directly to the food, contained in a separate container or incorporated into the packaging material (Espitia et al., 2012; Wen et al., 2016).

This has attracted much attention among demanding manufacturers and consumers, since essential oils and their derivatives have been classified as Generally Recognized As Safe (GRAS) by the US Food and Drug Administration. Some antimicrobial films containing essential oils and their derivatives have been evaluated for food packaging applications, such as polyethylene (PE), low-density polyethylene (LDPE), polypropylene (PP) and polyvinyl poly(vinyl chloride) films (Kuorwel et al., 2011).

The initial use of antimicrobial essential oils in food was through the addition of this direct on the product, however this caused some undesirable interferences in the final product, as cited by Silva, Domingues and Nerín (2016) where the addition of antimicrobials in chicken meat was realized by this way. Although still in use, the direct addition of antimicrobial compounds to poultry formulations may result in partial inactivation of the antimicrobial by interaction with the food matrix constituents, thus limiting its effects on food microbiota (Higueras et al., 2013).

Promising examples of active packaging incorporating essential oils or their constituents are compiled in Table 2.2, as previously mentioned. This packaging was applied to foods with the aim of extending the shelf life of the food while sustaining the nutritional and sensory qualities of the food. Active packaging incorporated with essential oils has already been applied to several foods, such as fresh beef, butter, fresh octopus, bologna, ham and fish (Salgado et al., 2013).

Essential oils, as well as their compounds, can be added into food or incorporated into packaging. Biobased food packaging has been largely used to incorporate essential oils to protect different types of food (e.g., meat, fish, fruits, processed and raw food) with effective results (Ribeiro-Santos et al., 2017). Essential oils or natural extracts of Rosmarinus officinalis L. are known to possess antimicrobial, and some applications of these extracts in food active packaging systems have been reported in the literature (Realini and Marcos, 2014).

As an example, rosemary active packaging was the most effective method to protect pork meat from lipid oxidation, compared to oxygen scavenger packaging (Bolumar et al., 2016). Another example was presented by Sirocchi et al. (2016), where the microbiological analysis of meat showed that the counts of psychrophilic, Brochothrix thermosphacta, Enterobacteriaceae and Pseudomonas spp. bacteria were lower in meat with active packaging than in meat with non-active packaging, especially in high-O2 conditions.

According to Vartiainen et al. (2014), the antimicrobial films can be produced using petroleum-based materials (low density polyethylene, polyethylene, polyvinyl chloride, among others) to provide packages or they can be biobased to be used as edible coatings (whey proteins, gelatin, chitosan, among others) or biopolymers for packaging (such as poly-lactic acid, polyhydroxyalkanoates, pectin and cellulose nanofibrils).

When applied to food packaging, Wen et al. (2016) demonstrated that polyvinyl alcohol/cinnamon essential oil/b-cyclodextrin (PVA/CEO/b-CD) antimicrobial nanofibrous film can effectively prolong

the shelf-life of strawberry, indicating its potential for the application in active food packaging.

Essential oils of oregano and clove incorporated in whey protein edible films showed that the films obtained were effective in controlling microbiota present on poultry samples (mesophilic, Enterobacteriaceae and Pseudomonas) at concentrations of 30 g/kg, with oregano essential oil being more effective than clove and with both essential oils yielding lower reductions in the number of Pseudomonas (Fernandez-Pan et al., 2013).

The preservation of food occurs by diffusion of the active compounds in the essential oil incorporated in the packaging to the food and may be influenced by several factors, such as type of essential oil, polymer, food composition, time and temperature of contact (Ribeiro-Santos et al., 2017).

The encapsulation of aromatic compounds in essential oils within gelatin capsules for potential application in beverage packaging was the subject of WO patent 2013032631 A1 (Zhang and Given, 2013). There is another patent regarding the production of biodegradable nanoparticles sensitive to variations in temperature and/or pH, formed from block copolymers composed of a hydrophobic, biodegradable segment of the polyester family, and that can be used for the controllable encapsulation of active molecules in foods to achieve their subsequent release into a specific medium under a stimulus specific (BRPI0902050) (UNIVERSIDADE DE SÃO PAULO; INSTITUTO DE PESQUISAS TECNOLÓGICAS DO ESTADO DE SÃO PAULO, 2011). The patent WO 2006000032-A1 (Suppakul, Miltz, Sonneveld and Bigger, 2003) regards an antimicrobial packaging material for foodstuffs containing 0.05 to 1.5% by weight of a natural essential oil. Incorporating additives in packaging instead of in the food can be a promising field of research.

An invention made by Nerín de La Puerta et al. (2009) reported in patent number WO2007144444-A1 relates to the development of a novel active packaging that inhibits food pathogens either by means of the generation of an active atmosphere or by means of direct contact, which comprises a support made from paper, cardboard, cork, aluminum or wood and an active coating therefore. The coating consists of a formulation of paraffin and natural plant extracts.

Also, Garcés and Nerin De La Puerta (2010) developed an antimicrobial packaging based on the use of natural extracts that consists of packaging to which a coating or an active liquid is applied composed of a basic formulation that consists in: a resin substrate made of nitrocellulose, acrylics, vinylics, etc, solvents and additives to confer

flexibility or other characteristics to the coating. The active liquid is comprised of resins, which are selected depending on the type of packaging, solvents that give the system fluidity, additives that confer it flexibility and, finally, of active components, which in this case are natural extracts with or without fixatives.

Wu et al. (2018) have shown a study on the influence of factors in the release of antimicrobial materials from active packaging, some of which are already commercialized. The ability of an antimicrobial package to extend the shelf-life of the food depends on the rate of release of the active compounds from the package, which is influenced by several factors such as: active compound type, food simulant, packaging materials, interactions between them, the temperature and relative humidity of the environment. According to these authors, few of the studies published on the subject have carried out research on real foods and therefore new research needs to remedy this deficiency. Despite all the weakness, the industrial-scale production of active antimicrobial food packaging is still promising.

The use of nanotechnology in food has the potential to revolutionize the food process, food resources and the way in which food is delivered once packaged and evaluated. Nanotechnology is also expected to allow us to develop new foods and new production methods. Because of this, the development of new additives with different food protection functions is of great scientific importance and technological interest.

2.5 Conclusions and future perspectives

Food preservation, quality maintenance and safety are growing concerns in the food industry due to the growing consumer demand for natural products and safe food and the implementation of increasingly stringent standards to prevent foodborne infectious diseases. The development of studies and the application of active packaging with the potential to improve food safety is a prerequisite for the future. Given the obvious differences between laboratory-scale and actual applications in food packaging, the time of release of antimicrobial agents is a very important factor and can mean the difference between life and death of a microorganism. Further studies are needed on the incorporation and protection of antimicrobial agents in food packaging films and the evaluations of all the implications for food safety. Based on this review, it is imperative to establish a multidisciplinary approach, bringing together experts from all areas of biotechnology, particularly

microbiology, food technology, engineering and materials science, to create a promising future for the use of antimicrobial agents in food packaging.

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3 MICROBIOLOGICAL CHARACTERIZATION OF PURE GERANIOL AND COMPARISON WITH BACTERICIDAL ACTIVITY OF THE CINNAMIC ACID IN GRAM-POSITIVE AND GRAM- NEGATIVE BACTERIA

This chapter is based on the paper entitled "Microbiological Characterization of Pure Geraniol and Comparison with Bactericidal Activity of the Cinnamic Acid in Gram-Positive and Gram-Negative Bacteria" (Journal of Microbial & Biochemical Technology, Volume 7(4): 186-193 (2015)), by Micheli Zanetti, Raquel Z Ternus, Francieli Dalcanton, Josiane M.M. Mello, Humberto Gracher Riella, Pedro H. H. de Araújo, Débora de Oliveira, Marcio Antônio Fiori. Abstract, keywords and acknowledgements were omitted.

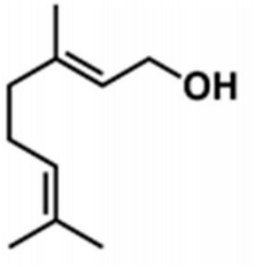
3.1 Introduction

In recent years, aromatic plants and their extracts have been examined for their effectiveness for food safety and preservation applications [1-3] and have received attention as growth and health promoters [4]. Most of their properties are due to their essential oils (EOs) and other secondary plant metabolite components [4]. Biological activities of EOs include, but are not limited to, antibacterial, antifungal, antiviral, insecticidal, antioxidant, and anti-cancer properties [5,6]. Essential oils have been documented to be effective antimicrobials against several foodborne pathogens including *E. coli* O157:H7, *Salmonella Typhimurium*, *S. aureus*, *L. monocytogenes*, *Campylobacter* and others [7]. The use of natural essential oils as antimicrobial agents in food products may be considered as an additional key to increasing the security and shelf life [8].

Many authors have performed studies in vitro on antibacterial properties on several Eos [9, 10] finding minimal inhibitory concentrations (MIC) values very low (< 0,1 %) against an initial concentration higher than 105 CFU mL^{-1} of many pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella Enteritidis*, *Campylobacter jejuni* and *Escherichia coli* [11].

Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene alcohol with the chemical formula $C_{10}H_{18}O$ (Figure 3.1). The product referred to as "geraniol" is a mixture of the two cis-trans isomers properly named geraniol (trans) and nerol (cis). Geraniol is a commercially important terpene alcohol occurring in the essential oils of several aromatic plants [12].

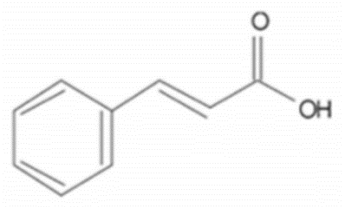
Figure 3.1 - Chemical structure of the geraniol.



Si et al. (2005) evaluated the antimicrobial activity of six types of essential oils and the geraniol showed a antimicrobial effect for the bacteria *Escherichia coli* and *Salmonella Typhimurium* greater than 80 % for 500 μg ml^{-1} oil concentrations [13]. These works obtained antimicrobial activity results of the palmarosa oil (80 % of geraniol in its composition) considered strong, and this oil showed MIC between 0.4 and 0.6 mg L^{-1} for *Staphylococcus aureus* and *Escherichia coli* [14]. The geraniol gas demonstrated the antimicrobial activity for the pathogens agent of the respiratory systems, including the bacteria *Haemophilus influenzae, Streptococcus pneumoniae, S. pyogenes* and *Staphylococcus aureus* [15].

The cinnamic acid, also known as 3-phenyl-2-propenoic acid (Figure 3.2) consists of a naturally occurring aromatic fatty acid originated from higher plants and found in Estoraques, cinnamon oils, and coca leaves [16], has low toxicity and a broad spectrum of biological activities against numerous microorganisms [17]. Cinnamic acid derivatives typically have a broad spectrum of biological activity including antioxidant, cerebroprotective, hepatoprotective, cholegogic, anti-inflammatory, cytostatic, antihypoxic, hypolipidemic, anticoagulant, antiallergic, antimicrobial, etc [18] and are widely used in food additives [19]. Cinnamic acid is the main constituent of clove oil composing approximately (70 to 80%) followed by the composition of eugenol (4 to 7%) [20].

Figure 3.2 - Chemical structure of the cinnamic acid.



Many cinnamic acid derivatives, especially those with the phenolic hydroxyl group, are well-known antioxidants and are supposed to have several health benefits due to their strong free radical scavenging properties. It is also well known that cinnamic acid has antimicrobial activity. Cinnamic acid derivatives, both isolated from plant material and synthesized, have been reported to have antibacterial, antiviral and antifungal properties [21].

Tests with cinnamic acid showed antibacterial activity against all the tested bacteria (gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC 7973), and *Micrococcus flavus* (ATCC 10240), and gram negative bacterium *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311) and *Enterobacter cloacae* (human isolate) with MIC 700-15000 μg/mL [22].

Many studies mention the antimicrobial activity of *Cymbopogon martinii*, commonly known the palmarosa or Rosa grass, that is a perennial aromatic grass, known for its rose like aroma essential oil and refer this activity to geraniol. The essential oil obtained from genuine *Cymbopogon martinii* is commonly known in trade palmarosa oil, which contains geraniol and geranyl acetate the major compounds [23]. Whole plant contains essential oil of palmarosa, but maximum amount of oil and geraniol are eeported in flowering tops [24]. The characteristic odor of palmarosa oil is attributed to the high content of geraniol and varying amounts of geranyl acetate [24].

Several authors refer to antimicrobial activity palmarosa to geraniol in that it is in higher concentration, but it is known that palmarosa extract is composed of various other oils and esters, including the geranyl acetate, which is also widely quoted in the literature as possessing antimicrobial activity [26, 23, 27]. Therefore, the need for further work, which can demonstrate antimicrobial activity of geraniol pure oil.

In this context, the aim of this study was to evaluate the minimum inhibitory concentration and the antimicrobial sensitivity of the essential oil of geraniol and cinnamic acid against different microorganisms *Staphylococcus aureus*, *Listeria monocytogeneses*, *Escherichia coli* and *Salmonella enterica*.

3.2 Material and methods

3.2.1 Microorganisms and inoculum preparation

The geraniol and cinnamic acid were obtained as analytical standards from Sigma Aldrich. Antibacterial activity tests were performed with standard Gram-positive strains, *Staphylococcus aureus* (ATCC 25923) (Bioscan) and *Listeria monocytogeneses*, gram- negative strains, *Escherichia coli* (ATCC 25922) (Bioscan) and *Salmonella enterica* (ATCC 14028) (Bioscan).

Using a sterile loop seeding the microorganisms were transferred to cultures on nutrient agar - AN (Kasvi) for brain-heart broth tubes - BHI (Kasvi) and incubated in a greenhouse bacteriological (Quimis, modelo Q316M4) with controlled temperature of 35 ± 1 ° C for 24 h. After the incubation process the bacterial suspensions were adjusted in sterile 0.9% saline (synth), in order to obtain an optical turbidity comparable to McFarland 0.5 standard solution with an approximate concentration of 10^8 CFU mL^{-1}. The turbidity was measured by a spectrophotometer (Biospectro, Model SP22) and transmittance signals at wavelength of 625 nm. Likewise, the bacterial suspension was diluted and adjusted to an approximate concentration of 10^4 CFU mL^{-1}

.

3.2.2 Diffusion Assay Agar

The antimicrobial activity of the geraniol and cinnamic acid was assessed using a diffusion test results with pit on solid medium (agar), following the method of susceptibility testing for antimicrobial agents described by the standard CLSI (2012) with modifications.

With the aid of sterile cotton swab, the bacterial suspensions of the different microorganisms (10^4 CFU mL^{-1}) were inoculated on the surfaces of Plate Count Agar - PCA (Kasvi). After 5 minutes, were made holes (wells) 5 mm in diameter and PCA with the aid of a micropipette (Brand) were filled with geraniol or cinnamic acid. The plates were incubated in bacteriological oven (Quimis, model Q316M4) controlled temperature of 35 ± 1 ° C. After 18 h. The diameters of inhibition zones were measured using a millimeter ruler. All agar diffusion tests were performed in triplicate for each organism.

3.2.3 Determination of minimum inhibitory concentration (MIC)

The determination of the minimum inhibitory concentration (MIC) was performed according to the procedures described by [28] with some modifications. The geraniol and cinnamic acid were initially diluted to a concentration of 10000 μg.mL^{-1} in aqueous solution containing 10% by volume of dimethylsulfoxide (DMSO - Vetec). The microdilution test was performed in sterile 96-well microplates shaped bottom "U" containing 100 μL of BHI (brainheart infusion broth). Then 200 μL of the diluted solutions geraniol and cinnamic acid was added to the wells. The tests were performed in triplicate and denominated by A1, A2, A3 samples containing cinnamic acid and A5, A6 and A7 for samples containing geraniol. After homogenization of the sample (line A) 100 μL aliquots of each solution were successively transferred to new microplates, to obtain bacterial solutions of different concentrations, Table 3.1.

Table 3.1 - Solutions with different concentrations of bacterial geraniol or cinnamic acid

Sample	Concentration of geraniol or cinnamic acid (μg mL^{-1})
A	10000.000
B	5000.000
C	2500.000
D	1250.000
E	625.000
F	312.500
G	156.250
H	78.125

In the solutions shown in Table 3.1 were added 5 μL of bacterial inoculum of *Staphylococcus aureus*, *Listeria monocytogeneses*, *Escherichia coli* and *Salmonella enterica*, prepared with a concentration of 10^4 CFU mL^{-1}. The coded solutions as samples 10, 11 and 12 were used as control solutions for experimental testing. The 10 code solutions were

used as negative control in the inhibitory activity of the DMSO solvent used in the preparation of all the bacterial solutions. Solutions containing 10% DMSO were diluted in Mueller-Hinton broth as the equivalent concentration of 78.125 mg L^{-1} (equivalent to H samples) and the same inoculum was added. The solutions coded as 11 samples were prepared containing BHI broth and only the inoculum in order to serve as positive control of bacterial viability. The coded solutions were prepared samples containing only Mueller-Hinton broth with the aim of verifying the sterility of the plate.

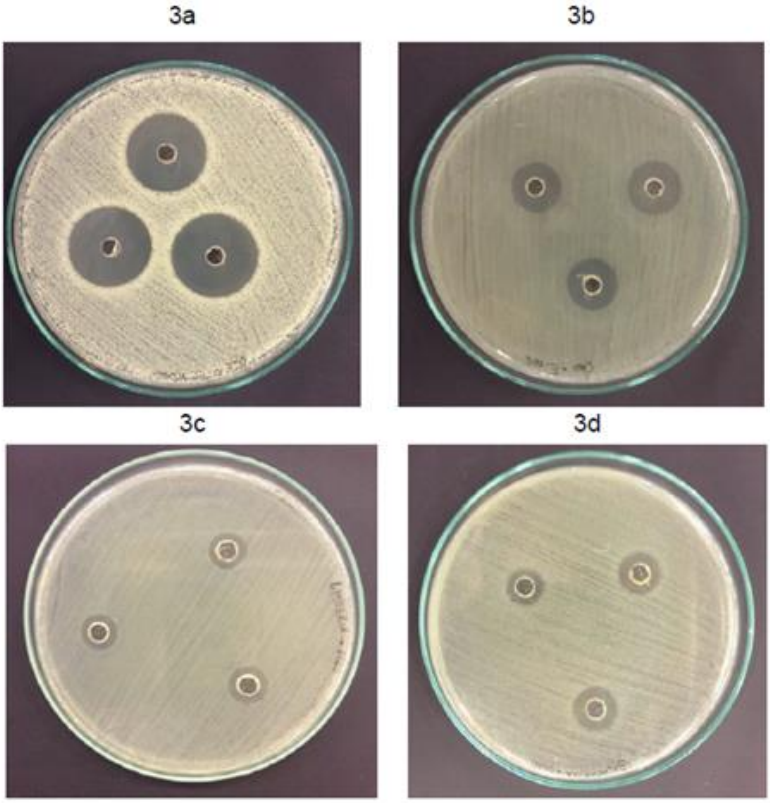
The microplates were incubated in a microbiological incubator with controlled temperature 35ºC for 20 h. After the incubation was added to each microplate 20 μL of an aqueous solution of 0.5% TTC (triphenyl tetrazolium chloride-Sigma-Aldrich) and the microplates were re-incubated for another 4 h. After the last incubation, the presence of the red coloration in the holes indicated microbial growth, while the absence of color indicated the inhibitory activity of the samples geraniol and cinnamic acid.

3.3 Results and discussion

3.3.1 Microbiological tests

Figure 3.3 shows the results of agar diffusion test for the pure geraniol oil with different types of bacteria and the Table 2 shows the average diameter of the halo inhibition measured for agar diffusion tests. The geraniol showed an excellent antimicrobial activity for the four bacteria tested and a bactericidal effect. In the agar diffusion test for bacteria type *Staphylococcus aureus* the halo average diameter of the inhibition zone was (35.3 ± 0.08) mm. For the bacteria *Escherichia coli*, the halo average diameter was (25.5 ± 0.05) mm and for *Salmonella enterica* the average diameter was (17.6 ± 0.15) mm. For *Listeria monocytogeneses* the average diameter was (15.8 ± 0.04) mm.

Figure 3.3 - Diffusion test results in agar for the geraniol with different bacteria. (a) *Staphylococcus aureus*, (b) *Escherichia coli*, (c) *Listeria monocytogeneses* e (d) *Salmonella enterica*.



According to the antimicrobial activity rating from agar diffusion tests established by Ponce et al. the geraniol is microbiologically very active compounds for the bacteria Staphylococcus aureus, Escherichia coli, Salmonella enterica and microbiologically active for the bacterium Listeria monocytogeneses [29]. The method establishes that average diameters of halos of inhibition smaller than 9 mm should be considered microbiologically not active; average diameters between 9 and 14 mm microbiologically active part; average diameters between 14 and 17 mm microbiologically active and higher average diameters 17 mm microbiologically very active.

Table 3.2 - Average diameter values for the inhibition halos in agar diffusion test for the geraniol and cinnamic acid using different types of bacteria: Staphylococcus aureus, Escherichia coli, Listeria monocytogeneses and Salmonella enterica.

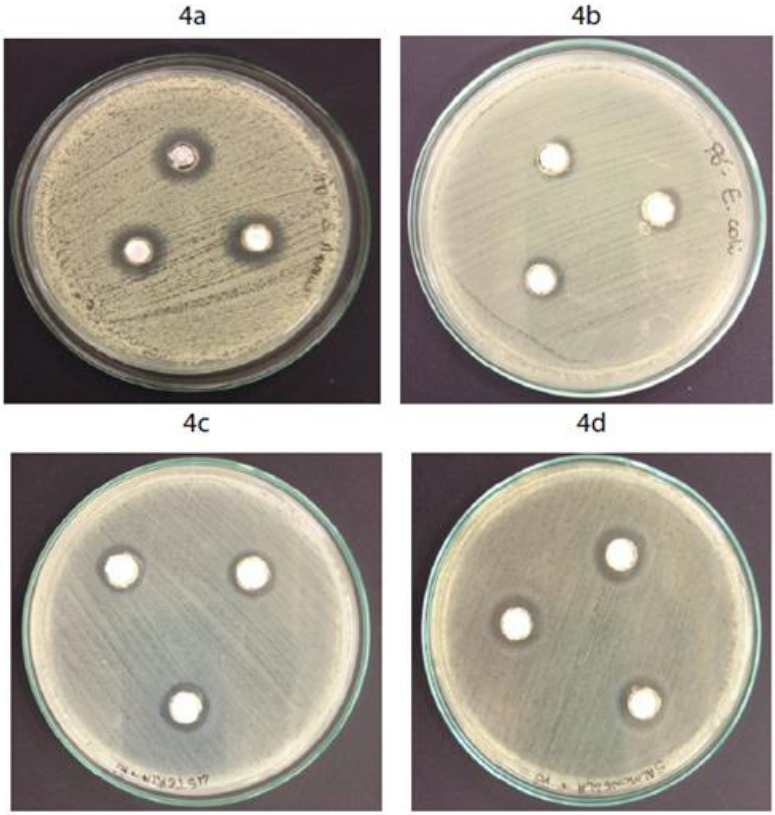
Samples	**Mean (± SD) diameter values for the inhibition halos (mm)**			
	Microorganisms			
	S. aureus	*E. coli*	*L. monocytogeneses*	*S. enterica*
Geraniol	35.3 ± 0.08	25.5 ± 0.05	15.8 ± 0.04	17.6 ± 0.15
Cinnamic acid	16.5 ± 0.10	11.0 ± 0.06	14.7 ± 0.12	14.8 ± 0.04

The cinnamic acid showed less antimicrobial activity than geraniol. The Figure 3.4 shows the results of microbiological tests by agar diffusion obtained for pure cinnamic acid and different types of bacteria. For bacteria of type *Staphylococcus aureus*, the average diameter of the inhibition halo was (16.5 ± 0.10) mm and for the bacteria of the type *Salmonella enterica* (14.8 ± 0.04) mm. For bacteria type *Listeria monocytogeneses* the average diameter of the inhibition halo was (14.7 ± 0.12) mm and for the bacteria type *Escherichia coli* the halo average diameter was (11.0 ± 0.06) mm. Following the classification of Ponce [29], cinnamic acid is a microbiologically active compound for the bacteria *Staphylococcus aureus*, *Salmonella enterica*, and *Listeria monocytogeneses*. For bacteria type *Escherichia coli* cinnamic acid is microbiologically active part.

In addition, essential oils are known to exhibit antimicrobial activity against a wide range of bacteria and fungi. The antimicrobial activity of essential oils is due to their solubility in the phospholipid bilayer of cellmembranes [30]. It was also reported that the antibacterial activities of monoterpene alcohols (including linalool, nerol, citronellol and geraniol) are more effective than their antifungal activity [31]. Assays have shown that geraniol demonstrates good activity in modulating drug resistance of several gram-negative bacterial species (*E. aerogenes*, *E. coli*, *P. aeruginosa*) by targeting efflux mechanisms and could restore drug susceptibility in strains that overexpress efflux pumps [32].

The results of the microbiological agar diffusion tests show that geraniol has antimicrobial activity against all types of bacteria studied. The cinnamic acid, despite having less antimicrobial activity also inhibits bacterial growth of the studied strains.

Figure 3.4 – Diffusion test results in agar for the cinnamic acid with different bacteria. (a) *Staphylococcus aureus*, (b) *Escherichia coli*, (c) *Listeria monocytogeneses* e (d) *Salmonella enterica*.



The greatest antimicrobial activity geraniol oil is probably due to its greater hydrophobicity. Some authors have shown that natural extracts have high hydrophobicity and therefore cross bacterial membranes with high easily, leading to loss of ions and reducing the electric potential of membranes and loss of function of protons and decreased ATP. These changes promote cell death of the bactéria [33].

Table 3.3 shows the results of the minimum inhibitory concentration test (MIC) for the four bacteria studied, the compounds held geraniol and cinnamic acid. The minimum concentration of geraniol

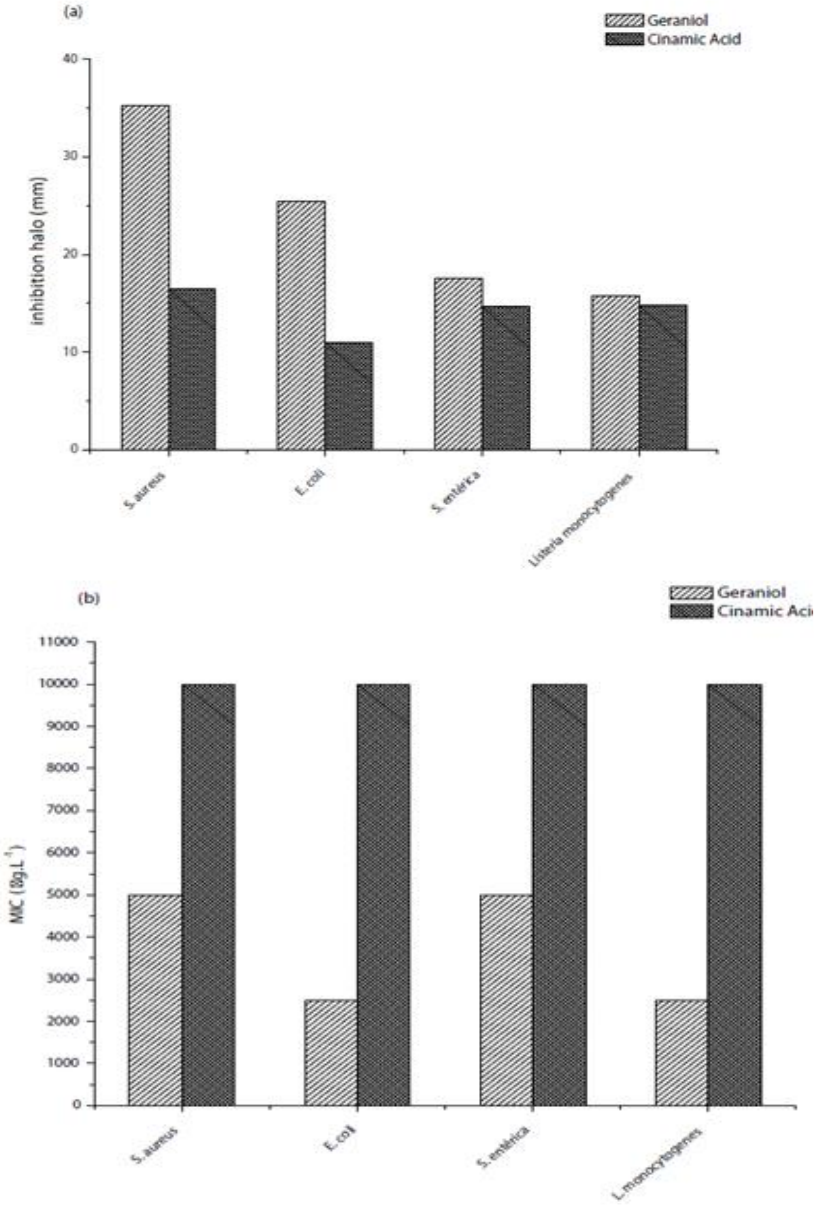
required for the inhibition of growth was lower for *Escherichia coli* and *Listeria monocytogeneses*, being 2500 µg mL^{-1}. For *Staphylococcus aureus* and *Salmonella enterica* concentration was 5000 µg mL^{-1}. Cinnamic acid a concentration of 10000 µg mL^{-1} was required to inhibit the growth of bacteria assessed, being a concentration at least twice that of geraniol. The minimum inhibitory concentration was not associated with the type of cell wall of bacteria, Gram-positive or Gram-negative.

Table 3.3 - Results of the Minimum Inhibitory Concentration test (MIC) for the geraniol and cinnamic acid using different bacteria: Staphylococcus aureus, Escherichia coli, Listeria monocytogeneses and Salmonella enterica.

Compound	**MIC (µg mL^{-1})**			
	S. aureus	*E. coli*	*L.monocytogenese*	*S. enterica*
Geraniol	5000	2500	2500	5000
Cinnamic acid	10000	10000	10000	10000

Figure 3.5 compares the microbiological results obtained for compounds of geraniol and cinnamic acid and is shown that geraniol has greater antimicrobial activity than cinnamic acid.

Figure 3.5 - Microbiological results of geraniol, cinnamic acid against bacteria S*taphylococcus aureus* and *Escherichia coli* (Fig. 3.5(a)), and the inhibition tests (Fig. 3.5(b)).



For the four types of bacteria evaluated geraniol showed greater antimicrobial activity of cinnamic acid in the agar diffusion test, Figure 3.5a, and the inhibition tests, Figure 3.5b.

3.3.2 Application options as antimicrobial

The tested bacteria are causing various diseases to humans and animals. The bacterium *Staphylococcus aureus* cause foodborne diseases and infections ranging from minor skin disorders localized to the deep tissue and life-threatening systemic disease. The bacteria *Escherichia coli* are facultative anaerobic and are associated with urinary tract infections and nosocomial infections in humans [32].

Salmonella enterica is associated with different clinical manifestations: gastroenteritis, bacteremia, enteric fever, and an asymptomatic carrier state and is more common in children under the age of 5, adults 20-30 year olds, and patients 70 years or older [34-36].

Listeria monocytogenes is a facultative anaerobic gram positive bacteria and capable of causing diseases in humans. According to the European Authority for Food Safety (EFSA), *L. monocytogenes* is considered a microorganism of concern because, although their incidence in the population is relatively low, remains in the body for a long time, causing the hospitalization people had 99.1% of the confirmed cases, with a mortality rate of 15.6% in 2013 [37]. In the US, were reported 103 cases of human listeriosis, of which 15 resulted in death, in 2011, all associated with *L. monocytogenes* [40]. Consequently, in recent decades, researchers have devoted intense efforts to decrease the incidence of this pathogen mainly in food and the use of geraniol and cinnamic acid may be an excellent alternative.

The results of this work show that the pure geraniol oil has great antimicrobial activity to the broad spectrum of pathogenic bacteria evaluated. Compared with cinnamic acid geraniol oil inhibits the growth and has higher antimicrobial activity for both Gram negative and Gram positive for bacteria.

These characteristics add various application possibilities for the oil geraniol. Its high antimicrobial activity and associated with the inhibition capacity of a large number of bacteria can be exploited for the development of antimicrobial technologies for food protection, control and prevention of infections in environments frequented by human beings or animals, or for the synthesis of new compounds with antimicrobial properties, as is the case of geraniol esters.

Nowadays, the approaches that can be adopted in food preservation include: (a) aseptic handling and packaging, (b) the mechanical removal

of microorganisms by washing or filtration, (c) destruction of microorganisms by physical or chemical sanitization and finally (d) the inhibition of pathogens or saprophytes through environmental control [41]. Recent developments in nanotechnology have brought significant advances in the development of active packaging using antimicrobial compounds in its composition.

Inhibition of microbial growth through environmental control is achieved through the addition of chemical compounds (antimicrobial preservatives) with an inhibitory or bactericidal/fungicide activity [42]. In the last years, natural antimicrobials have attracted considerable attention due to the increased consumer awareness on the aspects of food quality and safety [43, 44, 41].

Nanoencapsulation of bioactive compounds represents a viable and efficient approach to increasing the physical stability of the active substances, protecting them from the interactions with the food ingredients and, because of the subcellular size, increasing their bioactivity [42].

Several encapsulation systems, with different formulations and size distributions were tested for the delivery of essential oils in foods. For example, the use of a solid-fat micrometric emulsion was reported to reduce the volatility of the antimicrobial agent, entrapped into the crystallized fat structure, thus protecting the active compounds against environmental factors [45].

Some authors evaluated several EOs for antibacterial activity against the E. coli O157:H7 and Salmonella Hadar in apple juice. Compounds most active against E. coli included carvacrol, oregano oil, geraniol, eugenol, cinnamon leaf oil, citral, clove bud oil, lemon grass oil, cinnamon bark oil, and lemon oil. The most active compounds against S. Hadar were Melissa oil, carvacrol, oregano oil, terpeineol, geraniol, lemon oil, citral, lemon grass oil, cinnamon leaf oil, and linalool [46].

In addition to the acids most frequently added to melon and watermelon juices, essential oils have also been studied to improve the microbiological safety of these substrates [47]. Geraniol was added at concentration of 10 μg/mL to inactivate *Salmonella enterica* serovar *Typhimurium* completely (>8 log10 cycles) in melon juices (pH 5.91) [48].

The geraniol and cinnamic acid present many interesting biological properties, in addition to antimicrobial properties, which can be applied to the development of methodologies and technologies for the protection and conservation of food. These compounds are non-toxic, have antioxidant and anti-inflammatory activity. These properties associated

with high antimicrobial activity demonstrated by this study show the high application potential of these compounds and instigate new and further study of its application. Motivate studies to explore the consumer product formulation containing viability geraniol, cinnamic acid or its derivatives.

Likewise, motivate future studies to evaluate the technical feasibility of the application of production of antimicrobial additives, non-toxic, antioxidants and anti-inflammatory drugs with potential application in cosmetics, active packaging for foods, biological membranes and using physician.

3.4 Conclusions

The compounds tested, geraniol and cinnamic acid, exhibit activity against pathogens studied, Staphylococcus aureus, Listeria monocytogeneses, Escherichia coli and Salmonella enteric, being geraniol a compound that deserves attention for being very active for three of the four bacteria tested. Future research may focus on the efficacy of these compounds in various industrial applications. The synergism of these compound and processing techniques have to be investigated for potential commercial applications as a new additive for foods and packaging foods.

3.5 References

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4 SYNTHESIS OF GERANYL CINNAMATE BY LIPASE-CATALYZED REACTION AND ITS EVALUATION AS AN ANTIMICROBIAL AGENT

This chapter is based on the paper entitled "Synthesis of geranyl cinnamate by lipase-catalyzed reaction and its evaluation as an antimicrobial agent" (Journal of Chemical Technology and Biotechnology, v. 92, p. 115-121, 2017.), by Micheli Zanetti, Thaís Karoline Carniel, Alexsandra Valério, J Vladimir de Oliveira, Débora de Oliveira, Pedro H.H. de Araújo, Humberto Gracher Riella and Marcio A. Fiori. Abstract, keyworkds and acknowledgements were omitted.

4.1 Introduction

The production of flavors and fragrances through natural substances by an esterification process is a subject of scientific and industrial interest.[1–3] Owing to the fact that lipases (triacylglycerol ester hydrolases) may catalyze esterification reactions without providing high temperature and pressure conditions and since the procedure remains relatively uncomplicated when compared with chemical synthesis, such enzymes have acquired popularity as biocatalysts for reactions such as esterification. [4,5]

Esterification of alcohols is a major conjugative step in the terminal phase of the biosynthetic pathway of volatiles. It leads to enhanced volatility, modified olfactory properties, altered polarity as well as biological activities.[6,7] In recent years a great deal of research has been completed detailing the efficacy of plant-derived essential oil components (EOC) (e.g. thymol, carvacrol, allicin, geraniol, limonene, etc.) to inhibit the growth of food-borne bacterial pathogens.[8]

Geraniol (trans-3,7-dimethyl-2,6-oktadien-1-ol) is a primary, acyclic, doubly unsaturated terpene alcohol with a flowery, rose-like odor. It is naturally occurring in many oils like palmarosa, geranium or rose oils. It can undergo a number of reactions, such as rearrangement, cyclization or esterification. Owing to this, it is an interesting compound as an important intermediate in the production of other compounds with a wide range of applications.[8–10]

Cinnamic acid, also known as 3-phenyl-2-propenoic acid, consists of a naturally occurring aromatic fatty acid originated from higher plants and found in Estoraques, cinnamon oils, and coca leaves,[11] has low toxicity and a broad spectrum of biological activities against numerous

microorganisms.[12] Cinnamic acid is the main constituent of clove oil composing approximately 70 to 80% followed by eugenol (4 to 7%).[2] However, some essential oils are volatile, unstable to light and heat, with easy decomposition depending on the antimicrobial application.Generally, the esterification of some essential oils improves specific substrate properties such as emulsification, dispersion and overall quality of the consumer products.

Cinnamic acid derivatives typically have a broad spectrum of biological activity including antioxidant, cerebroprotective, hepatoprotective, cholegogic, anti-inflammatory, cytostatic, antihypoxic, hypolipidemic, anticoagulant, anti-allergic, antimicrobial, etc.[1] and are widely used in food additives.[13] Our research group has been working on antimicrobial natural compounds in recent years and identified geranyl cinnamate as a compound that can be used in the near future as an antimicrobial additive in food industries, for example. Chemical additives are always discussed by public organizations. Researchers in several parts of the world have studied the possible negative effects of these food compounds. In addition, it is possible to verify the microbial resistance to chemical products. This problem has led researchers to investigate and identify new natural and bioactive compounds. The compoundsynthesized here has the potential to be applied in food products.

In spite of the scientific and technological importance, there are few studies on production routes and evaluation of process parameters for the geranyl cinnamate ester, and no published studies that provide information about the antimicrobial and antioxidant properties of this compound was found in the open literature. Therefore, in this work, we have investigated the enzyme-catalyzed production and antimicrobial and antioxidant properties of geranyl cinnamate ester obtained fromesterification of geraniol and cinnamic acid.

4.2 **Materials and methods**

4.2.1 Materials

Candida antarctica lipase immobilized on a hydrophobic polymeric resin (NS88011) was kindly donated by Novozymes Brazil (Araucária, PR, Brazil). Cinnamic acid and geraniol (Sigma-Aldrich) and n-heptane PA (95%, Vetec) were used as substrates and solvent, without further purification.

4.2.2 Methods

4.2.2.1 *Geranyl cinnamate production*

In the preliminary step of this work an experimental design was used to evaluate the effects of important process variables on geranyl cinnamate production, following the ranges commonly used in the literature for lipase-catalyzed esterification reactions. Three parameters were investigated; enzyme concentration (10 to 20 wt%), temperature (50 to 90 °C) and geraniol to cinnamic acid molar ratio (1:1 to 5:1). The experimental design consisted of 11 experiments at three levels with replicates at the central point. Statistica® 8.0 (Statsoft Inc., USA) was used to assist the design and statistical analysis of experimental information, adopting a confidence level of 95% ($P < 0.05$).

The esterification reaction of cinnamic acid with geraniol was carried out in Erlenmeyer glass flasks (250 mL) using an orbital shaker (150 rpm), 10 mL of n-heptane as solvent and 2 h reaction time for all experiments. Control reactions without lipase were carried out in tandem with reactions using identical conditions. In order to purify the geranyl cinnamate and remove the unreacted reagents (enzyme, cinnamic acid, and geraniol) after all experiments the final reaction product was filtered and evaporated.

4.2.2.2 *Kinetic study of geranyl cinnamate synthesis*

A kinetic study was carried out in order to investigate the influence of immobilized *C. antarctica* lipase as catalyst in geranyl cinnamate synthesis. Concentrations of substrates were systematically varied over a wide range to study their effect on the rate of reaction using a range of immobilized *C. antarctica* lipases from 10 to 20 wt%. Different molar ratios of geraniol to cinnamic acid in the range from 1:1 to 5:1 were also studied. The effect of temperature on reaction kinetics was evaluated from 40 to 80 °C. The esterification reaction of cinnamic acid with geraniol was carried out in an Erlenmeyer glass flask (250 mL) using an orbital shaker (150 rpm), 10 mL of n-heptane as solvent and 6 h reaction time for all experiments.

In order to purify the geranyl cinnamate and remove the unreacted reagents (enzyme, cinnamic acid, and geraniol) after all experiments the final reaction product was filtered and evaporated.

4.2.2.3 *Geranyl cinnamate characterization*

Geranyl cinnamate esters produced were analyzed by gas chromatography (Shimadzu GC-2010) equipped with data processor, using a capillary fused silica INOWAX column (30m length × 250 micrometers i.d. × 0.25 micrometers thickness), flame ionization detector,with the following temperature program: 40–180 °C (3 °C.min^{-1}), 180–230 °C (20°C.min^{-1}), 230 °C (20 min), injector temperature 250 °C, detector at 275 °C, injection in split mode, split ratio 1:100, H_2 (56 kPa) as carrier gas, injected volume of 0.4 μL of diluted sample in n-hexane (1:10). The reaction conversion was calculated based on the reduction of geraniol peak area taken as limiting reagent in the esterification reaction.[14]

^{1}H-nuclearmagnetic resonance spectroscopy (^{1}HMNR) wasperformed in a Varian Gemini 300 spectrometer, operating at 300MHz using deuterated dimethylsulfoxide-d6 (DMSO-d6) as solvent at a concentration of 20mgmL^{-1}. Chemical shifts are given in parts per million from Me_4Si as internal standard. The relative areas of the signals were obtained by electronic integration and the chemical shifts (δ) are expressed in ppm relative to the solvent signal as well as the multiplicities (s=singlet, d=doublet, t=triplet, m=multiplet). For ^{1}H MNR analysis, the geranyl cinnamate ester sample was previously purified as already detailed.

4.2.2.4 *Antimicrobial activity of geranyl cinnamate*

Diffusion assay agar. The antimicrobial activity of the sample geranyl cinnamate esters was assessed using a diffusion test resulting in pitting of the solid medium (agar), following themethod of susceptibility testing for antimicrobial agents described by the standard CLSI (2012) with modifications.

With the aid of sterile cotton swabs, bacterial suspensions of the different microorganisms (10^4 CFUmL^{-1}) were inoculated on the surfaces of plate count agar (PCA; Kasvi). After 5 min, holes were made (wells) 5 mm in diameter in the PCA with the aid of a micropipette (Brand) and were filled with 100 μL of pure geranyl cinnamate. The plates were incubated in a bacteriological oven (Quimis, model Q316M4) at a controlled temperature of 35±1 °C. After 18 h, the diameters of the inhibition zones were measured using a millimeter rule. All agar diffusion tests were performed in triplicate for each organism.

Minimum inhibitory concentration (MIC). Determination of the minimum inhibitory concentration (MIC) was performed according to the

procedures described by Weerakkody[15] with some modifications. Samples of geranyl cinnamate esters were initially diluted to a concentration of 10.000 μgmL^{-1} in an aqueous solution containing 10% by volume of dimethylsulfoxide (DMSO - Vetec). The microdilution test was performed in sterile 96-well microplates with U-shaped bottom containing 100 μL of BHI (brain-heart infusion broth). Then 200 μL of the diluted solutions of geranyl cinnamate esters was added to the wells. The tests were performed in triplicate and denominated A1, A2, A3 for the samples containing geranyl cinnamate. In column A5 200 μL of BHI was added and in column A6 100 μL of a solution containing 10% by volume of dimethylsulfoxide (DMSO - Vetec) and 100 μL BHI was used; both columns were used as control. After homogenization of the samples, 100 μL aliquots of each solution were successively transferred to new microplates to obtain bacterial solutions of different concentrations. In columns A5 and A6 we observed bacterial growth, showing that DMSO at 10% did not cause bacteria death.

Next, 5 μL of bacterial inoculum of *Staphylococcus aureus* (ATCC 25923) (Bioscan) and *Escherichia coli* (ATCC 25922) (Bioscan), prepared with a concentration of 10^4 CFU mL^{-1} were added to the solutions. The samples coded 10, 11 and 12 were used as controlsolutions for the experimental testing. Sample 10 was used as a negative control in the inhibitory activity of the DMSO solvent used in the preparation of all the bacterial solutions.

Solutions containing 10% DMSO were diluted in Mueller-Hinton broth to the equivalent concentration of 78.12 mg L^{-1} (equivalent to H samples) and the same inoculum was added. The solutions coded 11 were prepared containing BHI broth and only the inoculum in order to serve as positive control of bacterial viability. The coded solutions were prepared with samples containing only Mueller-Hinton broth with the aim of verifying the sterility of the plate.

The microplates were incubated in a microbiological incubator at a controlled temperature 35 ∘C for 20 h. After the incubation time, 20 μL of an aqueous solution of 0.5% TTC (triphenyl tetrazolium chloride Sigma-Aldrich) were added to each microplate and the microplates re-incubated for another 4 h. After the last incubation the presence of red coloration in the holes indicated microbial growth, while the absence of color indicated inhibitory activity of the sample geranyl cinnamate esters.

4.2.2.5 *Antioxidant activity of geranyl cinnamate*

The antioxidant activity of geranyl cinnamate was carried out following Sousa et al.[16] and Lopes-Lutz et al.,[17] with modifications, using

a DPPH (1,1-difenil-2-picrilhidrazil) method. The antioxidant compounds present in the ester react with the methanol solution of DPPH, decreasing the absorbance as a function of ester concentration.

A solution of DPPH in methanol (40 μg mL^{-1}) was prepared and kept under refrigeration in the dark, and geranyl cinnamate at concentrations of 500; 1.000; 2.500; 5.000; 7.500; 10.000; 12.000 and 15.000 μg mL^{-1}) was added to the system. Reaction mixtures were prepared in tubes, adding 0.3 mL of sample solution and 2.7 mL of DPPH. The absorbance was measured by spectrophotometer Shimadzu UV-1601PC, at 517 nm, after 60 min reaction. The percentage of antioxidant activity was calculated by:

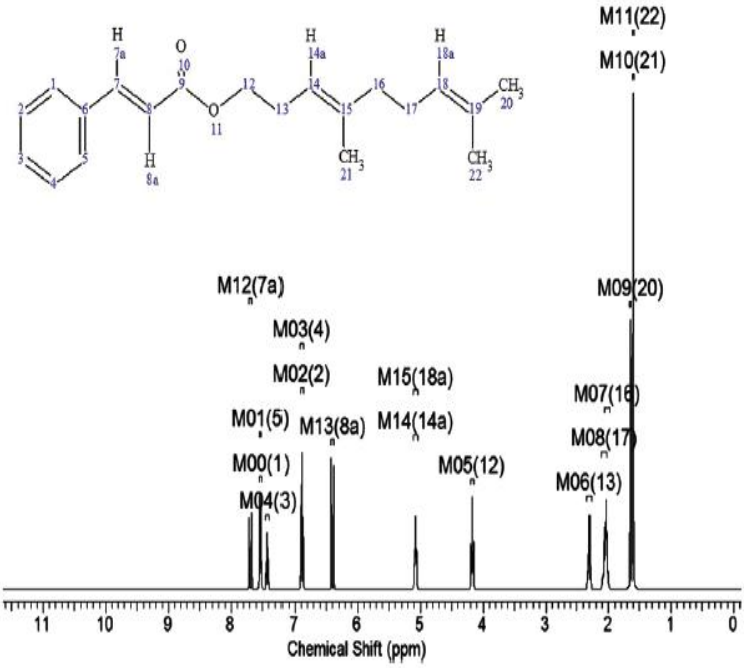
$$AA\% = [1- (\frac{A_{sample}}{A_{control}})] \times 1 \quad (1)$$

where A_{sample} is the absorbance of solution with the geranyl cinnamate and $A_{control}$ is the absorbance of control solution (2.7 mL of DPPH and 0.3 mL of methanol). Methanol was also used as blank.

4.3 Results and discussion

To confirm the synthesis of geranyl cinnamate ester ^{1}H NMR was performed. Figure 4.1 shows the ^{1}H-NMR spectrum obtained for geranyl cinnamate ester solubilized in deuterated chloroform. The ^{1}H-NMR spectrum shows characteristic peaks at δ 6.5–6.3 (m, 2H), 5.40–5.26 (t, 2H), 4.17 (t, 1H), 2.10–2.25 (m, 4H), 1.50–1.76 (m, 7H), and 1.0–1.45 (m, 5H).

Figure 4.1. ^{1}H MNR spectrum of geranyl cinnamate synthesized from geraniol and cinnamic acid catalyzed by immobilized *C. antarctica* lipase.



4.3.1 Evaluation of the synthesis of geranyl cinnamate ester

From the matrix of experimental design (Table 4.1) it was possible to observe that the best result in terms of geranyl cinnamate ester production was obtained at the central point under reaction conditions 70 °C, 15 wt% of enzyme and 3:1 geraniol to cinnamic acid molar ratio. Statistical analysis (data not shown) showed that all variables had a significant influence on geranyl cinnamate production. Enzyme concentration and geraniol to cinnamic acid molar ratio showed a positive effect, while temperature in the studied range showed a negative effect on ester conversion.

Table 4.1. Experimental design matrix (real and coded value) with response in terms of ethyl cinnamate conversion by immobilized C. antarctica lipase. Experimental conditions: 70 °C, 150 rpm, and 2 h reaction time

Run	Geraniol to cinnamic acid molar ratio (mol:mol)	Enzyme concentration (wt%)	Temperature (°C)	Conversion (%)
1	1:1 (-1)	10 (-1)	50 (-1)	6.54
2	5:1 (+1)	10 (-1)	50 (-1)	30.14
3	1:1 (-1)	20 (+1)	50 (-1)	33.31
4	5:1 (+1)	20 (+1)	50 (-1)	36.88
5	1:1 (-1)	10 (-1)	90 (+1)	19.78
6	5:1 (+1)	10 (-1)	90 (+1)	17.92
7	1:1 (-1)	20 (+1)	90 (+1)	18.65
8	5:1 (+1)	20 (+1)	90 (+1)	23.97
9	3:1 (0)	15 (0)	70 (0)	75.85
10	3:1 (0)	15 (0)	70 (0)	74.25
11	3:1 (0)	15 (0)	70 (0)	74.07

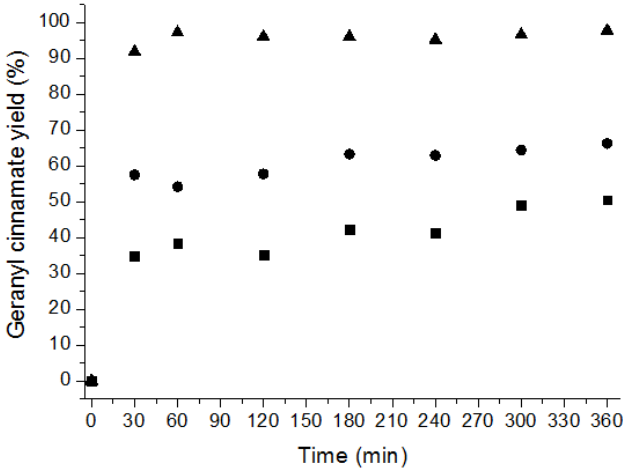
The influence of temperature obtained via the experimental design in this work revealed that when the temperature was increased to 90 °C a significant reduction in geranyl cinnamate was reported. High temperatures can be associated with enzyme denaturation, and as reported in thiswork can negatively influence the synthesis of geranyl cinnamate. For this reason, the temperature in the next step of this work was decreased.

Since the aim of this work was to improve geranyl cinnamate production and optimize the reaction time, a kinetic study based on the previous results obtained in the experimental design was performed. Therefore, the factors used for the kinetic study were temperature in the range from 40 to 80 °C, enzyme concentration in the range 10 to 20 wt%, and geraniol to cinnamic acid molar ratio from 1:1 to 5:1.

4.3.2 Effect of substratemolar ratio on the enzymatic kinetic of geranyl cinnamate synthesis

The effect of molar ratio geraniol to cinnamic acid on the lipase catalyzed esterification reaction was investigated. Figure 4.2 shows the conversions of esterification reactions, monitored over the course of 6 h of reaction time. Overall, increasing molar ratio geraniol to cinnamic acid from 1:1 to 5:1 gave an increase in geranyl cinnamate conversion from 38 to 97%, respectively, after 1 h reaction time. Furthermore, for all molar ratios studied in this work, reaction achieved a stationary state (plateau) of geranyl cinnamate yield after 30 min reaction time.

Figure 4.2. Effect of substrates molar ratio on lipase-catalyzed esterification of cinnamic acid with geraniol in n-heptane: (■) 1:1, (●) 3:1, and (▲) 5:1. Reaction conditions: 15 wt% of immobilized C. antarctica lipase NS88011 enzyme, 150 rpm, and 70 °C.



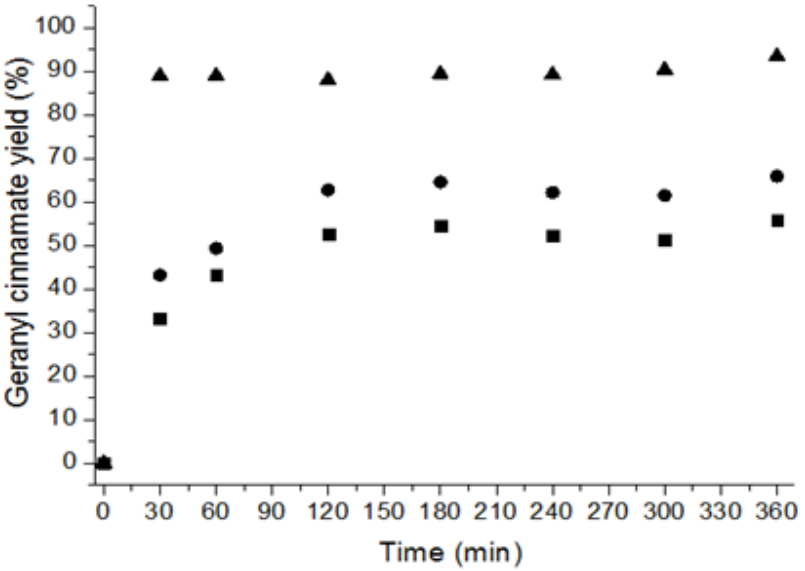
Similar results were reported by Paroul et al.[18] who studied the effect of geraniol to propionic acid molar ratio (1:1, 3:1 and 5:1) on geranyl propionate production, with reactions carried out at 50 °C, 10 wt% of immobilized enzyme *Candida antarctica* (Novozym 435) and 150 rpm. The authors reported that molar ratio influences the reaction conversion, reaching 99% of geranyl propionate conversion for molar ratio 5:1 after 6 h. Yun et al.[19] using lipozyme TL IM-catalyzed synthesized ethyl cinnamate through esterification of cinnamic acid with ethanol and showed an increase from 72.6 to 95.7% when substrate molar ratio was varied from 1:1 to 1:6. Mohamad et al.[20] under the optimum reaction conditions of 55 C, solvent n-heptane, geraniol and propionic acid with molar ratio of 5:1 and a reaction time of 6 h, using *Candida rugosa* lipase adsorbed onto multi-walled carbon nanotubes reported 51.3% production of geranyl propionate.

4.3.3 Effect of temperature on the kinetic of enzymatic synthesis of geranyl cinnamate

The effect of the reaction temperature was investigated through incubation of reaction mixtures at different temperatures ranging from 50 to 80°C, taking into account the results obtained earlier. As shown in Figure 4.3, the best conversion of geranyl cinnamate (93%) was obtained

at 80 °C. On the other hand, low conversion was observed when 40 and 60 °C was used. The results showed that temperature did not negatively affect the stability and the enzymeactivity, and positively affected synthesis rate untilreaching equilibrium. The synthesis rate increased with higher temperature; at 40 and 60 °C equilibrium was observed after 120 min reaction time, while at 80 °C equilibrium was observed after 30 min reaction time.

Figure 4.3. Effect of reaction temperature on lipase-catalyzed esterification of cinnamic acid with geraniol in n-heptane: (■) 40 °C, (●) 60 °C, and (▲) 80 °C. Reaction conditions: 15 wt% of immobilized *C. antarctica* lipase, 150 rpm, and 3:1 molar ratio geraniol:cinnamic acid.



Increasing temperature reduces the viscosity of the medium and favors the interactions between enzyme and substrate, so the reaction rate and reaction conversion increases. Increasing temperature can also increase the kinetic energy of molecules following the Arrhenius concept of activation energy enhancing the products formation.[14,21]

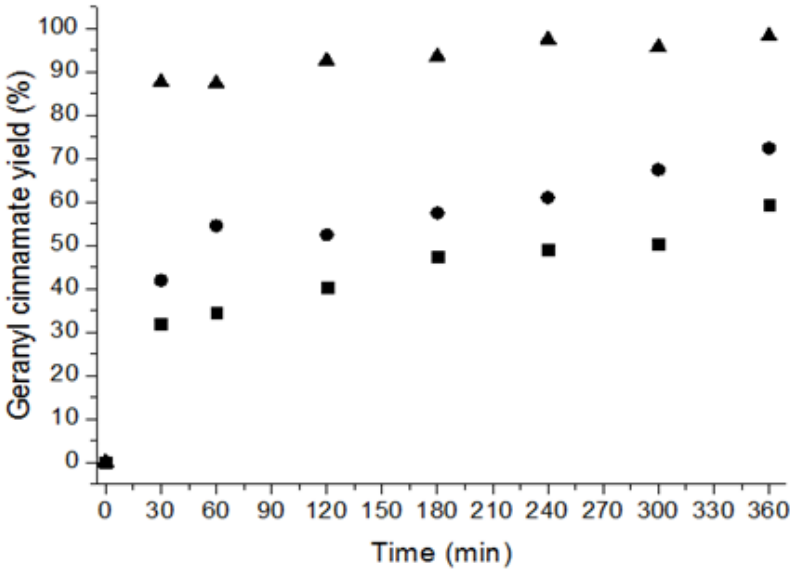
Chaibakhsh et al.[22] reported the optimization of enzymatic eugenol ester synthesis by statistical approaches. Eugenol and caprylic acid were used as substrates and lipozyme TL IM as catalyst. The maximum conversion yield (72.2%) was obtained under optimal conditions of 65

∘C, 250 rpm, 259 min, 100mg enzyme, and 2:1 molar ratio of eugenol to caprylic acid in a solvent-free system.

4.3.3.1 *Effect of enzyme concentration on the kinetic of enzymatic synthesis of geranyl cinnamate*

The effect of enzyme concentration on geranyl cinnamate conversion was evaluated at 70 ∘C keeping the molar ratio between geraniol and cinnamic acid constant at 3:1 and mixing rate at 150 rpm. The kinetic curves obtained are presented in Figure 4.4. From the results it can be seen that increasing enzyme concentration from 10 to 20wt% an increase in geranyl cinnamate conversion was observed. The maximum conversion achieved with 10 wt% of immobilized *C. antarctica* lipase was 21% after 6 h reaction time, lower than obtained with 20 wt% of immobilized *C. antarctica* lipase under the same conditions that achieve a higher geranyl cinnamate conversion (98%) in the same reaction time. In addition, it was possible to observe from the results, that using up to 15wt% of immobilized *C. antarctica* lipase, the system demands a longer reaction time, more than 6 h, to achieve the same range of geranyl cinnamate conversions as suggested by the curves of 10 and 15 wt% enzyme concentration shown in Fig. 4.4.

Figure 4.4. Influence of immobilized *C. antarctica* lipase concentration on lipase-catalyzed esterification of cinnamic acid with geraniol in n-heptane: (■) 10 wt%, (●) 15 wt%, and (▲) 20 wt%. Reaction conditions: 70 °C, 150 rpm, and 3:1 molar ratio geraniol:cinnamic acid.



4.3.3.2 *Antimicrobial activity of geranyl cinnamate*

Figure 4.5 shows the results of microbiological tests using agar diffusion for geranyl cinnamate synthesized in the optimum reaction conditions previously obtained and different types of bacteria. The geranyl cinnamate showed excellent antimicrobial activity for the two bacteria and a bactericidal effect. In the agar diffusion test for bacteria of type *Staphylococcus aureus* the inhibition zone had an average diameter halo of 22.8±0.40 mm. For bacteria of *Escherichia coli* type, the zone of inhibition had an average diameter of 20.2±0.41 mm, as shown in Table 4.2. The methods establish that average diameters of halos of inhibition smaller than 9 mm should be considered microbiologically not active; average diameters between 9 and 14 mm microbiologically partly active; average diameters between 14 and 17 mm microbiologically active and average diameters higher than 17 mm microbiologically very active.[23]

Figure 4.5 - Diffusion test results agar for geranyl cinnamate with different bacteria. (a) *Staphylococcus aureus* and (b) *Escherichia coli.*

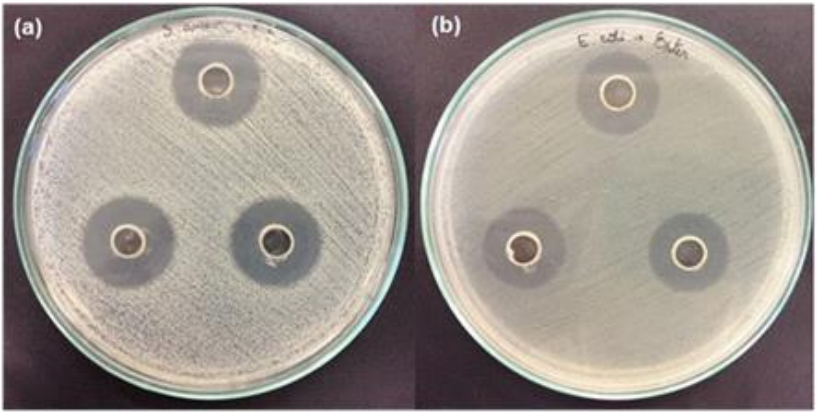


Table 4.2 - Average diameter of inhibition halos by agar diffusion for the compound geranyl cinnamate esters, evaluated with different types of bacteria.

Sample	Mean (± SD) diameter of the inhibition halos in mm		
	Microorganisms		**Reference**
	S. aureus	*E. coli*	
Geraniol	35.3 ± 0.08	25.5 ± 0.05	[24]
Cinnamic acid	16.5 ± 0.10	11.0 ± 0.06	[24]
Geranyl cinnamate	22.8 ± 0.40	20.2 ± 0.41	This work

It is possible to observe that the inhibition zone for the geranyl cinnamate for both bacteria presented an intermediate diameter compared to geraniol and cinnamic acid. This combination demonstrates the synergic effects of these two compounds.

The geranyl cinnamate proved to be a very active compound against gram positive and gram-negative bacteria and has the potential for use in additives technology. In nanoencapsulated form it can be applied as a natural antimicrobial additive in packages to increase the shelf life of foods.

Table 4.3 shows the results of the minimum inhibitory concentration test (MIC) for the two bacteria tested here. The minimum

concentration was the same as that found in thework of Zanetti et al.[24] for *Staphylococcus aureus* bacteria for the essential oil, geraniol, and two times lower than the concentration of cinnamic acid. For *Escherichia coli* the minimum concentration was twice that found in the work of Zanetti et al.[24] for geraniol, and half of the required concentration for cinnamic acid.

Table 4.3 - Results of minimum inhibitory concentration test (MIC) for geranyl cinnamate ester for bacteria *Staphylococcus aureus* and *Escherichia coli*.

Sample	MIC (μg/mL)		Reference
	S. aureus	*E. coli*	
Geraniol	5000	2500	[24]
Cinnamic acid	10000	10000	[24]
Geranyl cinnamate	5000	5000	This work

Figure 4.6 compares the results of the microbiological results found by Zanetti et al.[24] for compounds: cinnamic acid, geraniol, and geranyl cinnamate ester, produced in this work. We can see that the geranyl cinnamate ester also has high antimicrobial activity, almost equal to geraniol. Compared with cinnamic acid, the ester was more active against the bacteria analyzed, Fig. 4.6(a), and inhibition tests, Fig. 4.6(b).

4.3.4 Antioxidant activity of geranyl cinnamate

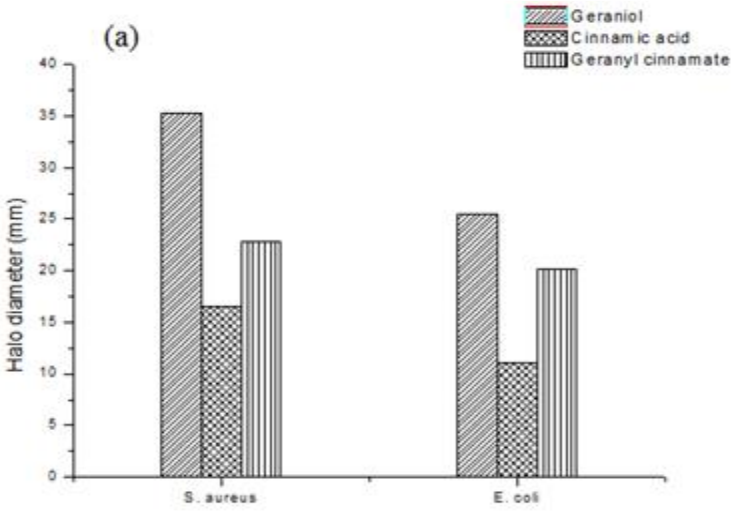
The quantitative evaluation of antioxidant activity (AA%) of geranyl cinnamate, at concentrations of 500; 1.000; 2.500; 5.000; 7.500; 10.000; 12.000, and 15.000 μg mL^{-1}, determined by DPPH assay, is presented in Table 4.4. The results show that the compounds do not have the sequestering activity of DPPH radical, as the sample of higher concentration (15.000 μg mL^{-1}) presented an antioxidant activity of only 8.49%, a low expected value for substances designed as antioxidants.

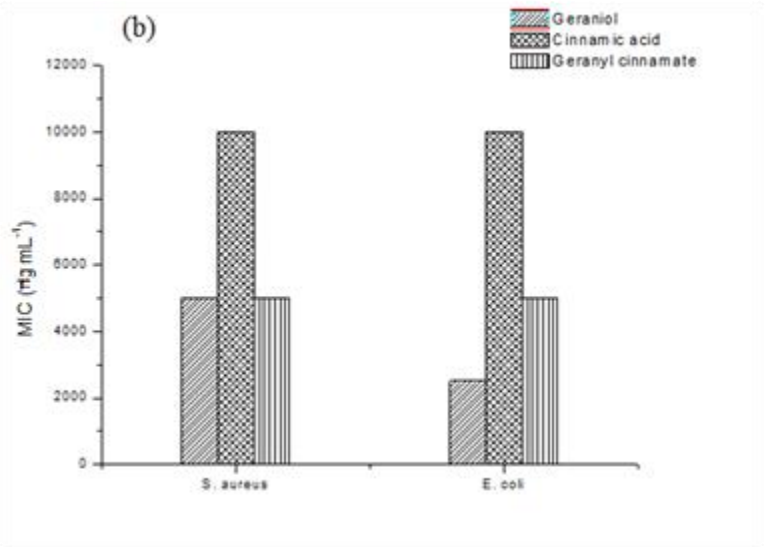
Table 4.4 - Percentage of neutralization of DPPH of geranyl cinnamate ester.

Concentration (μg/mL)	(AA%)
500	0.24
1000	0.73
2500	2.34
5000	4.25
7500	5.27
10000	6.39
12000	7.08
15000	8.49

Correlation between the antioxidant activity (%) and the ester concentration ($AA\% = 6E-06 + 0.0061$, R^2 =0.9534 gave an IC^{50} of 8.33 g mL^{-1}. The concentration of geranyl cinnamate necessary to reach 50% antioxidant activity. This concentration can be considered high concentration when compared with data from Cansian et al.[25] for other antioxidant substances, such as ascorbic acid (IC_{50} =2.15 μg mL^{-1}) and BHT (IC_{50} =5.37 μg mL^{-1}).

Figure 4.6 - Microbiological results of geraniol, cinnamic acid and geranyl cinnamate against bacteria *Staphylococcus aureus* and *Escherichia coli* (Figure 4.6a), and the inhibition tests (Figure 4.6b).





4.4 Conclusions

New experimental data on enzymatic esterification of geraniol and cinnamic acid for geranyl cinnamate production are reported in this work. From a kinetic study, the operating conditions that maximized geranyl cinnamate production were determined as geraniol to cinnamic acid molar ratio of 5:1, 70 °C, 150 rpm and 20 wt% of immobilized *C. antarctica* lipase, leading to a reaction conversion of about 97%. The results showed the promise of the technique to overcome thewell-known drawbacks of the chemical-catalyzed route. The compound exhibited activity against the pathogens studied, *Staphylococcus aureus* and *Escherichia coli*, so that geranyl cinnamate is a compound that deserves further attention for being very active for both bacteria tested. The compound showed no significant antioxidant activity.

4.5 References

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5 NANOCAPSULATION OF GERANYL CINNAMATE ESTER SYTHETIZED BY NEW ENZYMATIC PROCESS BY POLYCAPROLACTONE POLYMER

This chapter is based on the paper entitled “Nanocapsulation of geranyl cinnamate ester sythetized by new enzymatic process by polycaprolactone polymer” (submitted in Materials Science and Engineering: C), by Micheli Zanetti, Alessandra C. de Menezes, Debora de Oliveira, Pedro H. H. de Araújo, Luciano L. Silva and Marcio A. Fiori. Abstract, keyworkds, and acknowledgements were omitted.

5.1 Introduction

Natural extracts (NE) and essential oils (EO) have been studied by industrial sector, especially by food industries, for a long time due to their antimicrobial and antioxidant properties. Several phytochemical compounds present in the NE and EO are antimicrobial agents and can be effective in a variety of applications by reducing growth and survival of microorganisms [1]. The EOs are considered a natural preservative due to their inherent nature, and are frequently used in food products because they meet the current requirements of the consumers that prefer foods without synthetic preservatives [2].

Synthetic food chemical preservatives can be harmful to the human health and may provide numerous gastric problems [3]. These characteristics have increased the concern and interest of researchers for studies and applications of natural compounds (NE and EO) with the equivalent function in food. So, the NE and EO compounds have been used as natural antimicrobial compounds in foods and well-accepted by most consumers and also by the food industries.

The use of compounds with preservatives properties in industrialized foods is required to control the growth of bacteria and fungi, and to increase the shelf life of the produtcts. However, many studies indicate that some preservatives compounds can cause cancer and allergies in humans [4]. Despite health hazards, the truth is that these preservatives are essential to prolong the storage time of the foods. Therefore, the high challenge for the food industries is to use preservatives compounds that are not nocive for the human health and still have a good antimicrobial action. In this scenario, the use of antimicrobial natural extracts and antimicrobial essential oils is an attractive option.

Natural antimicrobials can be obtained from different sources, including plants, animals, bacteria, algae and fungi. Several studies with antimicrobials compounds obtained from plants have been demonstrating the efficacy of these compounds when applied to food preservation and the main factors that migh influence their effectiveness. Baṇkn et al. [5] suggested that green tea extract, and grape seed extracts delayed microbial spoilage, redness loss, and lipid oxidation, in low sulfite beef patties, thereby increasing shelf life. Raybaudi-Massilia, Mosqueda-Melgar and Martin-Belloso [6] studied that EOs of lemongrass, cinnamon, and geraniol were found to be effective in inhibiting the growth of *Salmonella enteritidis*, Escherichia *coli*, and *Listeria innocua*. Another study related that herbs and spices containing essential oils (EOs) in the range of 0.05-0.1% demonstrated activity against pathogens, such as *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, in food systems [7].

The essential oils interact with the cells of the microorganisms breaking the plasma membrane, damaging the cytoplasm, and in certain situations, altering the morphology completely, with consequent cell death [8, 9]. In particular, geraniol essential oil has a high inhibition capacity in Gram-positive and Gram-negative bacteria, as well as some types of fungi and yeasts [10].

The interest for the application of the NEs and EOs as additive in food packaging also has been growing. The possibilility of incorporation of these antimicrobial natural compounds directly in the polymeric films or in the cellulose generates good possibilities for the production of antimicrobial food packaging that are not toxic.

Antimicrobial packagings are used to decrease, inhibit and retard the growth of pathogenic microorganisms and to protect the food. The most of antimicrobial packagings are produced utilizing silver nanoparticles or glass microparticles doped with ionic silver as antimicrobial compounds [11-13]. These traditional additives generally are toxic in low concentrations and their use in antimicrobial packaging may cause great concern by the consumers. Consequently, the use of the NEs and EOs as nontoxical additive for the antimicrobial packaging is a great opportunity for minimizing these problems.

The use of the NEs or EOs as antimicrobial or antixodant additives is particularly challenging for the food industries or for the packaging industries. These compounds are very sensitives to temperature of the the manufacturing process and, frequently, are not missible in the polymer matrix. These characteristicis can be responsible for the thermodegradation and/or poor dispersion of the NEs or EOs on

the food packagings polymeric surface [14,2]. However, previous reports showed the enhancement of polymer properties are not only depended on the type and concentration of essential oil, but also on the compatibility between polymer matrix and essential oil [15].

An interesting alternative for protection against thermodegradation processes and increased missibility is the encapsulation of the NEs or EOs within another polymer with low melting temperature and missible in nonpolar matrix. The polymeric coating protects the natural compounds from thermodegradation during processing and favor the missibility in the polymeric matrix. In this way, the encapsulation of the NEs or EOs is an interesting option to the development of natural additives with high antimicrobial efficacy for food packagings.

The poly-ε-caprolactone (PCL) is an excellent polymer for the encapsulation of the NEs and EOs compounds for food applications as antimicrobial additive [16]. Polycaprolactone (PCL) is a semicrystalline aliphatic polyester, considered a biocompatible, and bioresorbable material with high permeability to drugs [17], and is approved by the FDA for food applications [18, 19]. This polymer has a low glass-transition temperature of -60 °C, a melting point of 60 °C, and exhibits high decomposition temperature (~ 350 °C).

The possibility of processing the PCL as a conventional thermoplastic material, added to its high solubility in a large number of organic solvents allows the production of verydifferent devices, like electron spun fibers, nanospheres, porous scaffolds, among others [20]. It has been shown that some controllable factors, such as polymer composition and molecular weight as well as temperature and pH, strongly influence the polymer degradation rate, reflected by a decreasing in the polymer molecular weight [21, 22]. Polymers can degrade and erode via surface or bulk process [23]. In relation to PCL nanoparticles some characteristics as size, shape and zeta potential, strongly influence the degradation and release of the active compounds [24]. Moreover, encapsulation techniques using PCL have already improved the heat stability [25].

PCL was used by Sosa et al. [26] to microencapsulate green tea polyphenols, and 3-5 μm microcapsules were obtained and the release prolongued for 90 h. Limayem Blouza et al. [27] encapsulated olive oil in PCL obtaining capsules of nanometric size from 320 to 400 nm. In a recent work, Zanetti et al. [28] related an interesting new antimicrobial ester derived from cinnamic acid syntetized by enzymatic reaction. Results showed that this ester has a high potential as antimicrobial

additive for food packagings. Several esters compounds are important non-toxic aroma used in the food industries and have been studied by the scientific and industrial community due to their antioxidant and antimicrobial properties. The geranyl cinamate is an example of ester, although little known in the literature, that presents a good antimicrobial and antioxidant properties [29]. Zanetti et al. [28] obtained geranyl cinamate via transesterification reaction of cinnamic acid with geraniol using immobilized *Candida antarctica* lipase. The antimicrobial activity of the geranyl cinamate was evaluated on bacteria *Staphylococcus aureus* and *Escherichia coli*. The ester showed excellent antibacterial activity for the two bacteria.

Thus, the viability of nanoencapsulation of the geranyl cinnamate ester using the polycaprolactone, PCL, as the encapsulating agent was evaluated and the effects of some important parameters, such as temperature and chemical composition of the release medium, were studied.

5.2 **Experimental Procedures**

5.2.1 Preparation of the geranyl cinnamate loaded PCL nanoparticles

The geranyl cinnamate ester was obtained through the esterification reaction of geraniol and cinnamic acid, following the methodology previously described by Zanetti et al. [28]. The ester was encapsulated in PCL nanoparticles by miniemulsification/solvent evaporation technique, the technique is based on the use of two immiscible solvents, the polymer and the ester being soluble in one (organic phase) and insoluble in the other, usually water. In this work, 0.300 g of polycaprolactone-PCL (Mw 2000 $g.mol^{-1}$) was dissolved in 10 g of dichloromethane CH_2Cl_2 (PA 99.5%) under magnetic stirring at 30 ºC for 1 h. The organic phase was composed of a 1:3 mass ratio of ester mass to polymer mass. The aqueous phase was composed of a solution containing 24 mL of waterand 0.01% SDS, and the aqueous phase was added under moderate magnetic stirring to the organic phase. The dispersion was then sonicated and left shaking for 24 h at 30 °C for evaporation of the organic solvent. After evaporation, nanoparticles were kept for 2 h at -80 ºC and freeze-dried at -50 ºC under 5 mtorr (9.67×10^{-5} psi) vacuum for 48 h in a Liotop L101 (Liobras - Brasil). D(+) trehalose (P.A.VETEC) was added to the nanoparticles dispersion before precooling at a ratio of 0.5:1 (w/w) relative to the amount of

nanoparticles. Finally, the lyophilized samples were stored in desiccator until further use.

5.2.2 Characterization of geranyl cinnamate loaded PCL nanoparticles

5.2.2.1 *Mean Particle Size (Dp), Polydispersity Index (PDI) and Zeta Potential (ZP)*

The average diameter (in intensity) and polydispersity index of the nanoparticles were determined by Dynamic Light Scattering (DLS) using the Zetasizer Nano S ZEN1600 (incident beam angle of 173° and wavelength of the 633 nm laser), from Malvern Instruments. The analysis were made at 25 °C from an aliquot of the miniemulsions without prior dilution.

Zeta potential was determined by Laser Doppler Anemometry associated with microelectrophoresis using the Zetasizer Nano ZS 3600 equipment, Malvern Instruments. Analysis were conducted with previous dilution in distilled water (1:10) at 25 °C.

5.2.2.2 *Nanoparticles Morphology Characterization*

The morphology characterization of the PCL nanoparticles loaded with geranyl cinnamate was performed by Transmission Electron Microscopy (TEM) using a JEM-1011 TEM (100 kV). The nanoparticles dispersions were diluted in distilled water up to 0.1% of solids; in sequence, one drop of each diluted sample was placed on a carbon-coated grid and dried under room conditions overnight.

5.2.2.3 *Thermogravimetric Analysis (TGA)*

To analyze the thermal stability of the PCL nanoparticles of geranyl cinnamate thermogravimetric analysis (TGA) was performed using a Shimadzu thermogravimetric analyzer (Model TGA 50) with a temperature range from 25 to 810 °C and a heating rate of 10 °C min^{-1} under nitrogen atmosphere with a flow rate of 50 $mL.min^{-1}$.

5.2.2.4 *Fourier Transform Infrared Spectroscopy (FTIR)*

A FT-IR spectrophotometer (Cary 600 Series FTIR from Agilient Technologies) was used to examine the chemical functional groups of the geranyl cinnamate, polycaprolactone and of the geranyl cinnamate loaded PCL nanoparticles. The absorption spectra of lyophilized nanoparticles were determined using a conventional KBr pellet method. Samples were

grinded and compressed with anhydrous KBr to form pellets. FTIR spectra were obtained in a range of 400 to 4000 cm^{-1} wave numbers.

5.2.2.5 *Differential Scanning Calorimetry (DSC)*

Lyophilized nanoparticles samples were analyzed using a DSC 4000 Perkin Elmer, under inert atmosphere (N_2, 20 $mL.min^{-1}$), from -20 to 90 °C at heating rate of 5 $°C.min^{-1}$. The thermal history was removed prior to the analyses at a heating rate of 20 $°C.min^{-1}$ and cooling rate of -20 $°C.min^{-1}$. The melting temperatures were thus obtained from the second heating run to investigate the influence of the geranyl cinnamate on the thermal behavior of the nanoparticles.

5.2.2.6 *Encapsulation efficiency of geranyl cinnamate*

For determination of the encapsulation efficiency, aliquots of a solution containing the nanoparticles were centrifuged at 14000 rpm for 90 min, under 4 °C. The nanoparticles containing the ester were left at the bottom of the eppendorfs and an aliquot of the supernatant was removed for analysis by UV/Vis spectroscopy at 273 nm using a UV-Vis (Merck, model Pharo 300) spectrophotometer. The encapsulation efficiency (EE%) was determined for three different geranyl cinnamate:PCL mass ratios: 1:1 (FLN 1), 1:3 (FLN 2) and 1:10 (FLN 3).

The concentration of free geranyl cinnamate was calculated using a calibration curve following Equation 1 and the ester EE% was determined using Equation 2. Equation 1 was obtained from calibration curves obtained experimentally for solutions with different concentrations. The geranyl cinnamate compound was diluted in dichloromethane (PA - Dynamic) at different concentrations. The absorption spectrum was performed in the range of 190 to 800 nm to check the maximum absorption wavelength for geranyl cinnamate.

In order to perform this analysis, the Beer-Lambert law was observed, where one of the real limitations is the concentration of the compound, high concentrations can result in linear deviations and changes in the refractive index, which can also be caused by noise in the detector. Thus, the measured absorbance of approximately 0.1 to 0.8 is ideal to avoid deviations from the law [30].

$$Abs = 92.526 * C_e - 0.046 \quad (1)$$

where, *Abs* is the absorbance and C_e is the geranyl cinnamate concentration ($\mu g.mL^{-1}$), following a correlation coefficient of $R^2 = 0.99$.

$$EE\% = \left(\frac{C_{initial} - C_{free}}{C_{initial}}\right) x100 \quad (2)$$

where, $C_{initial}$ is the amount (μg) of free oil not encapsulated dispersed in water determined from Equation 1 and C_{free} is the total amount (μg) of geranyl cinnamate added to the initial emulsion.

5.2.2.7 *Stability of nanoparticles*

The stability of the geranyl cinnamate loaded PCL nanoparticles in water was evaluated during 60 days, measuring the pH of the dispersions, the mean particle size, polydispersity index, the zeta potential and the amount of geranyl cinnamate that remained in the nanoparticles. All aqueous dispersions containing the nanoparticles were kept at 4 °C. The pH of the samples was measured with calibrated pHmentro, model Quimis-Q400AS.

5.2.3 Evaluation of antimicrobial activity of geranyl cinnamate loaded PCL nanoparticles.

5.2.3.1 *Diffusion assay agar*

The antimicrobial activity of the geranyl cinnamate loaded PCL nanoparticles was evaluated for *Staphylococcus aureus* (ATCC 25923) (Bioscan) and *Escherichia coli* (ATCC 25922) (Bioscan) bacteria. The tests were performed using a diffusion test with pit on solid medium (agar), following the method of susceptibility testing for the antimicrobial agents described by the standard CLSI with modifications. The bacterial suspensions of the different microorganisms (10^4 CFU mL^{-1}) were inoculated on the surfaces of Plate Count Agar - PCA (Kasvi) using sterile cotton swabs. After 5 minutes, holes (wells) of 5 mm of diameter were made at the PCA, and filled with geranyl cinnamate loaded PCL nanoparticles. The plates were incubated in bacteriological oven (Quimis, model Q316M4) controlled temperature of 35 ± 1 °C. After 18 h the diameters of inhibition zones were measured using a millimeter ruler. All agar diffusion tests were performed in triplicate for each microrganism.

5.2.3.2 *Minimum inhibitory concentration (MIC)*

The determination of the Minimum Inhibitory Concentration (MIC) was performed according to the procedures described by Weerakkody et al. [31] with some modifications. A mass of 0.05 g of geranyl cinammate loaded PCL nanoparticles were initially diluted to a

concentration of 5 μg.mL^{-1} in water and an aqueous solution containing 10% in volume of dimethyl sulfoxide (DMSO - Vetec). The micro dilution test was performed in sterile 96-well microplates shaped bottom "U" containing 100 μL of BHI (brain-heart infusion broth). Then 200 μL of the diluted solutions was added to the wells. After homogenization of the sample in the line Aa, 100 μL aliquots of each solution were successively transferred to the next well in the column, to obtain solutions with different concentrations. The PCL nanoparticles solutions were diluited to the concencentrations showed in Table 5-1, then, 5 μL of bacterial inoculum of *Staphylococcus aureus* and *Escherichia coli* was added to each solution. Negative and positive control samples were prepared using, respectively, DMSO and BHI broth. A solution was prepared containing only Mueller-Hinton broth to verify the sterility of the plate.

Table 5-1 - Bacterial solutions with different concentrations of geranyl cinnamate in PCL nanoparticles with 1:3 geranyl cinnamate:PCL mass ratio used in the MIC tests.

Sample	Concentration of geranyl cinnamate in PCL nanoparticles (μg.mL^{-1})
A	3.33
B	1.66
C	0.83
D	0.41
E	0.21
F	0.10
G	0.05
H	0.03

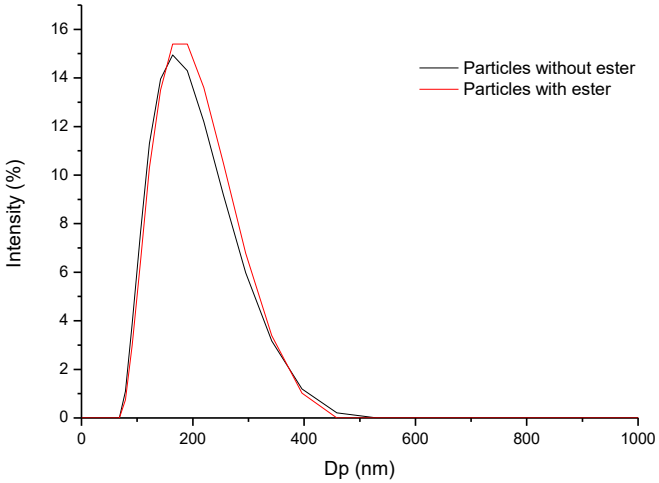
The microplates were incubated in a microbiological incubator with controlled temperature at 35 ^{o}C during 20 h. After the incubation 20 μL of an aqueous solution of 0.5% TTC (triphenyl tetrazolium Chloride-Sigma-Aldrich) was added to each microplate and then the microplates were re-incubated for another 4 h. After the last incubation, the presence of the red coloration in the wells indicates microbial growth, while the colorless indicates an inhibitory activity of the solutions containing the geranyl cinnamate loaded PCL nanoparticles. The tests were performed in triplicate.

5.3 Results and Discussion

5.3.1 Mean Particle Size (Dp) and Polydispersity Index (PdI)

Figure 5.1 shows the mean particle size (Dp) and polydispedity index (PdI) obtained by DLS for the PCL nanoparticles synthesized with 1:3 mass ratio of geranyl cinnamate:PCL and without geranyl cinnamate. The PCL nanoparticles synthetized without geranyl cinnamate had mean size of 168.8 nm and PdI of 0.165. The geranyl cinnamate loaded PCL nanoparticles presented an average size of 177.6 nm and PdI of 0.173. These results indicated that the presence of geranyl cinnamate did not affect significantly the particle size distribution, and the PdI bellow 0.2 indicates a good homogeneity in the particles size distribution.

Figure 5.1 – DLS results of particle size distribution of PCL nanoparticles without geranyl cinnamate and with 1:3 geranyl cinnamate: PCL mass ratio.

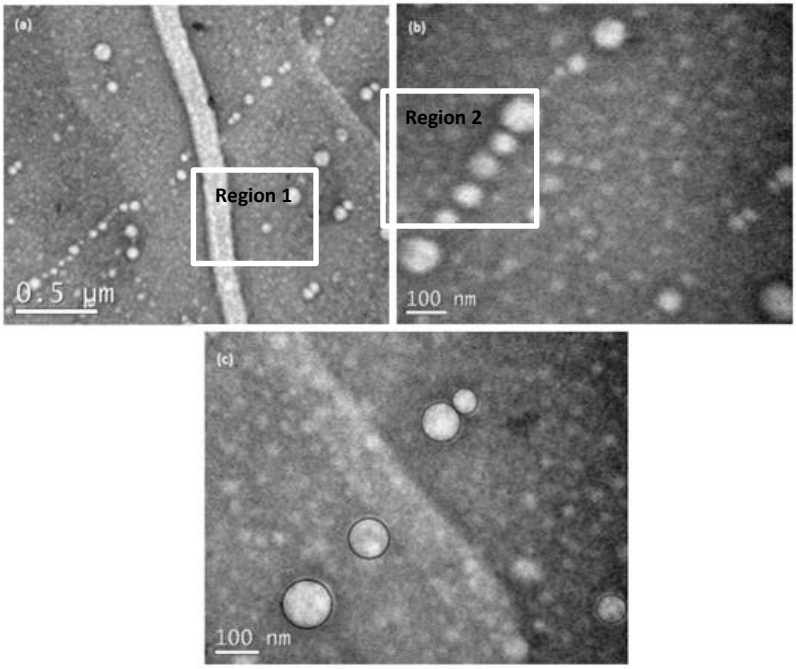


5.3.2 Nanoparticles morphology characterization

The morphology of the geranyl cinnamate loaded PCL nanoparticles were analyzed by TEM micrographs and are showd in the Figure 5.2. The Figure 5.2a shows the formation of nanoparticles with spherical format and with similar diameters of those measured by DLS. Figure 5.2a was amplified in two regions, and Figure 5.2b and 5.2c show

the region 1 and 2, respectively. The amplified micrographs revealed details of the nanoparticles spherical shape with diameter around 100 nm.

Figure 5.2 (a) TEM images of PCL nanoparticles synthetized with geranyl cinnamate (1:3 geranyl cinnamate: PCL mass ratio). (b) Details of the Region 1 with spherical nanoparticles and diameter size around 100 nm. (c) details sharpest of the particles and with better visualization.



5.3.3 Thermogravimetric Analysis (TGA)

The TGA thermograms presented in Figure 5.3 were obtained for geranyl cinnamate, PCL and geranyl cinnamate loaded PCL nanoparticles. The profiles of the first derivative of the TGA curve provide detailed information about the weight loss behavior. The geranyl cinnamate TGA thermogram presented a peak in the first derative curve at 169 °C that correspond to the maximum weight loss rate (Figure 5.4b). For PCL, the peak in the first derivative curve was 404ºC (Figure 5.4c). The same peak at the same temperature was observed for the decomposition of PCL in geranyl cinnamate loaded PCL nanoparticles (Figure 5.4a).

Figure 5.3 - TGA thermograms obtained for geranyl cinnamate, PCL and the geranyl cinnamate loaded PCL nanoparticles (1:3 geranyl cinnamate: PCL mass ratio).

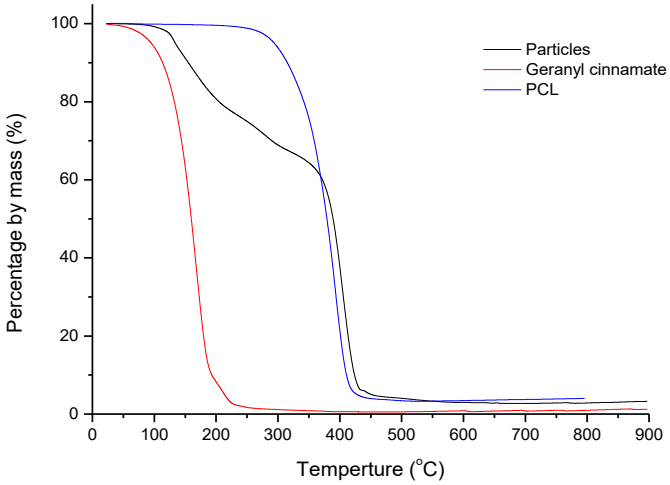
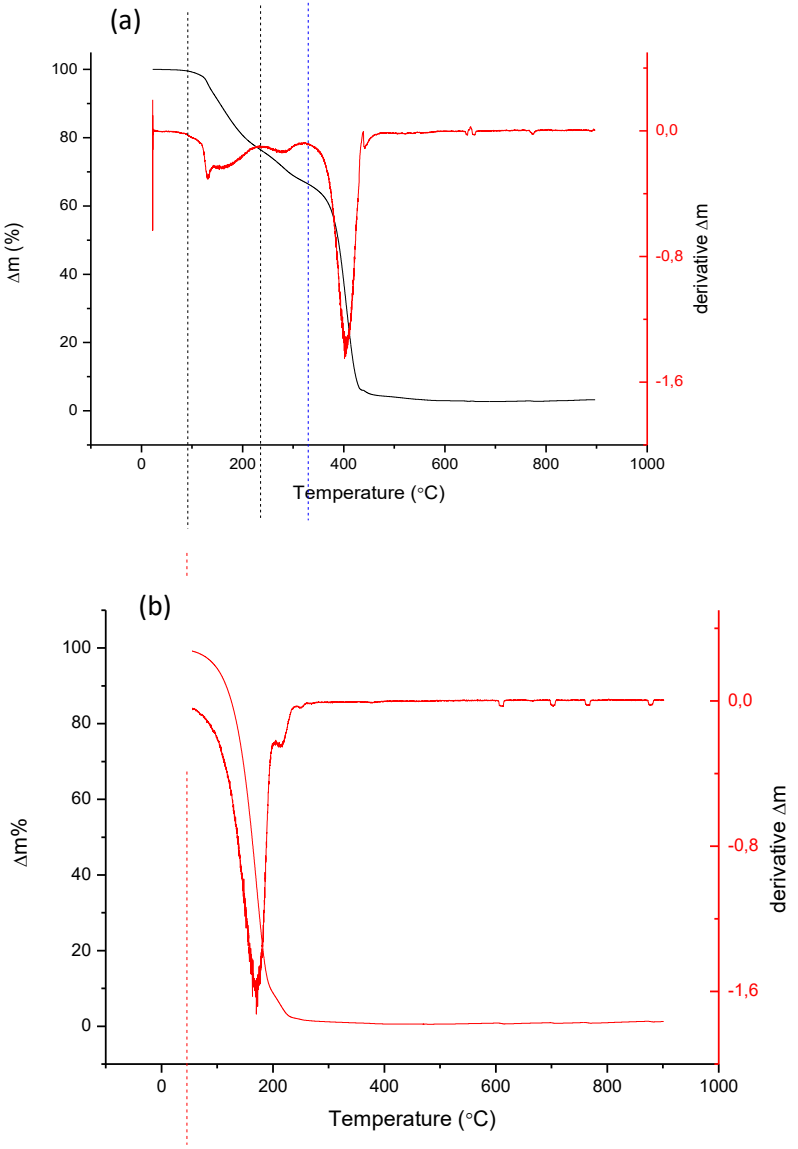
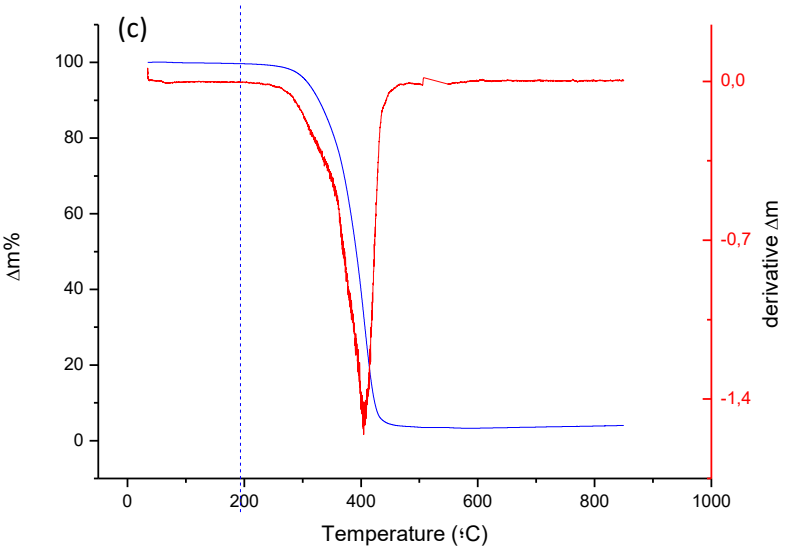


Figure 5.4 - TGA profiles for geranyl cinnamate loaded PCL nanoparticles (1:3 geranyl cinnamate: PCL mass ratio). (a) geranyl cinnamate (b) and PCL (c).





However, two distinctive peaks appeared at lower temperatures related to volatilizarion and degradation of geranyl cinnamate, at 128 ºC and 218 ºC. This behavior suggests that part of the geranyl cinnamate (not entrapped in the polymer matrix) volatilizes just like the free geranyl cinnamate, whereas the ester entrapped in the PCL nanoparticles remains in the polymer matrix and begins to degrade only when the polymer begins to decompose.

5.3.4 Infrared Spectroscopy with Fourier Transform (FT-IR)

FT-IR analysuswas used to evaluate the presence of charcateristics functional groups in the geranyl cinnamate, PCL, geranyl cinnamate loaded PCL nanoparticles, as well as possible modifications in the spectras due to the interactions between PCL and ester molecules. The FTIR spectras are shown in Figure 5.5.

Figure 5.5 - FT-IR spectra of geranyl cinnamate, PCL and geranyl cinnamate loaded PCL nanoparticles (1:3 geranyl cinnamate: PCL mass ratio).

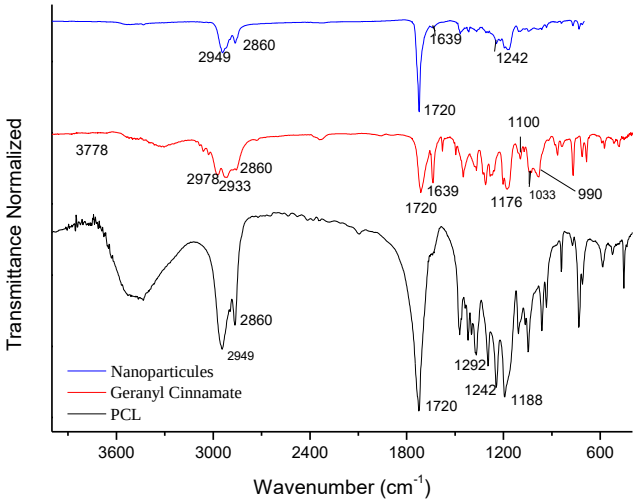
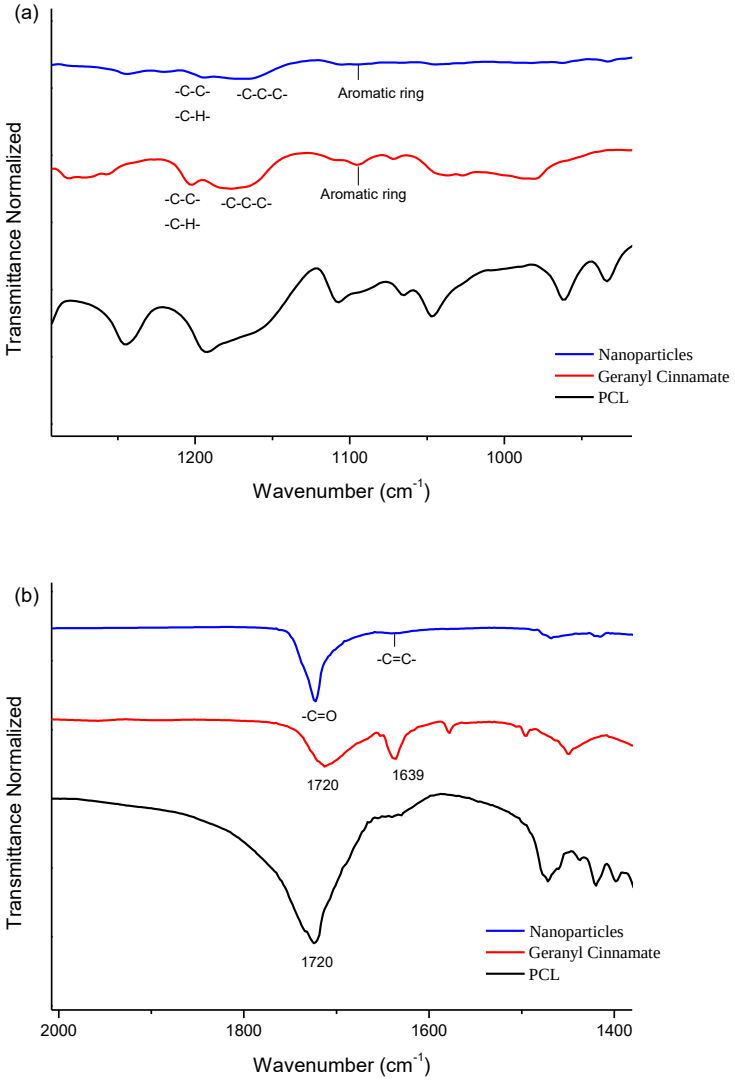


Figure 5.6 - FTIR spectra of geranyl cinnamate, PCL and geranyl cinnamate loaded PCL nanoparticles in the range of 800 to 1300 cm^{-1} (a) and 1500 to 1900 cm^{-1} (b).



FTIR spectrum obtained for PCL is in accordance with previosly reported by Elzein et al. [32] and Elzubair et al. [33]. Some bands in the

spectrum are strong, such as the band relative to the vibrational mode of the stretching of the carbonyl group at 1720 cm^{-1}. The bands relative to the vibrational modes associated with the CH_2 groups are shown at 2849 and 2860 cm^{-1} and the relative broadband to the vibrational modes of OH groups. According to Coleman and Zarian [34], the band at 1294 cm^{-1} is assigned the C-C and C-O bonds of the PCL molecule main chain stretching modes and has been used in the literature to investigate the change in crystallinity of PCL.

FTIR spectrum for the geranyl cinnamate is composed of functional groups derived from cinnamic acid, for example the presence of a band in 1033-990 cm^{-1} that indicates the presence of the aromatic ring and the peak in 1710 cm^{-1} the presence of the binding C=O. Some important groups are notice in the region 3326 cm^{-1} and 1416 cm^{-1} related to the OH, in the region of 2978 and 2933 cm^{-1} suggesting CH_2 functional groups, and in the region 1022 cm^{-1}, 946 cm^{-1} and 863 cm^{-1} indicating the carbon-carbon (C-C) bonds [35]. Figure 6a presents a detailed evaluation of the FTIR spectra in the range of 800 to 1300 cm^{-1} and in the range of 1500 to 1900 cm^{-1} in Figure 6b.

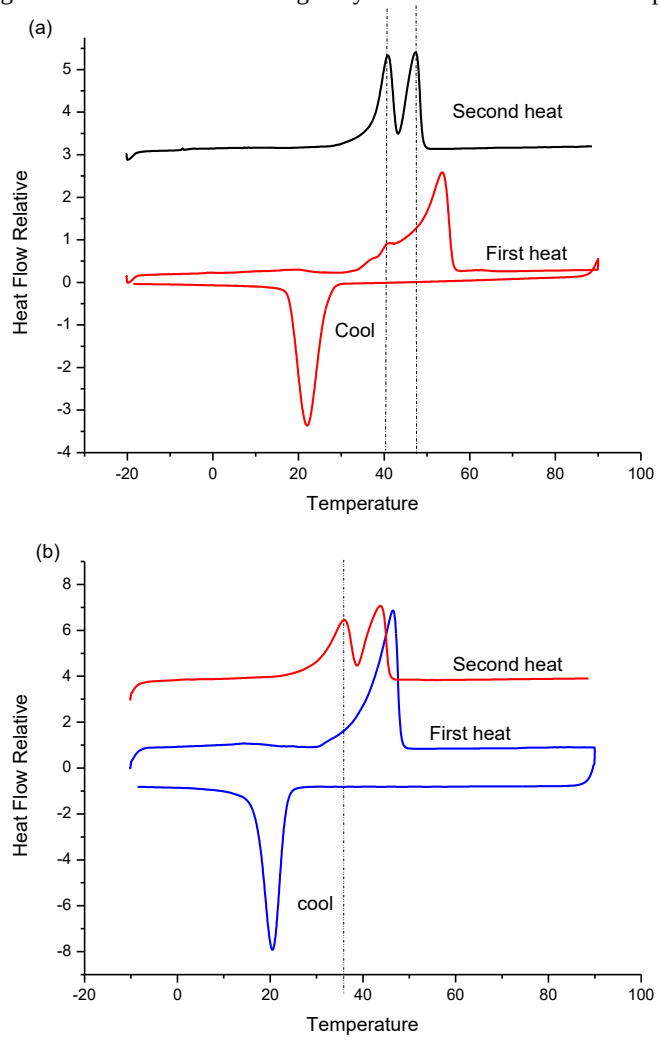
The FTIR spectra obtained for the geranyl cinnamate loaded PCL nanoparticles did not show any evidence of significant displacement of the main transmittance bands when compared to the isolated substances profiles. Although the characteristic bands of the PCL nanoparticles loaded geranyl cinnamate presented a lower intensity when compared to the pure compounds, the characteristic bands of pure geranyl cinnamate ester and pure PCL were observed, confirming the encapsulation of the ester in the nanoparticles polymeric matrix.

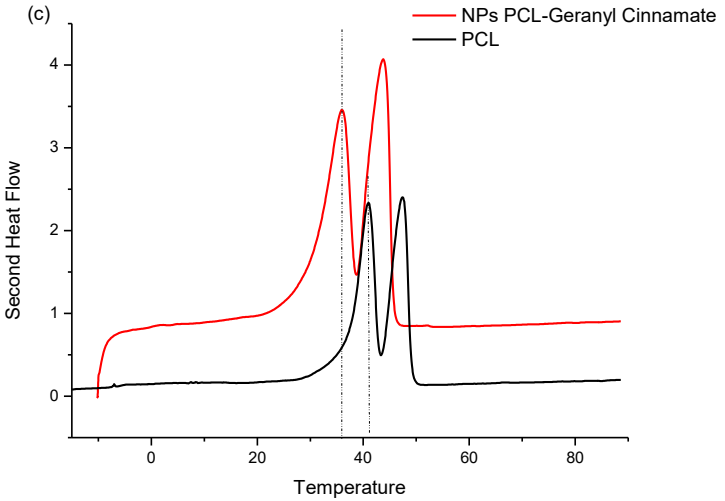
5.3.5 Thermal analysis by differential Scanning Calorimetry (DSC)

Thermal analyzes for the dry samples were performed by DSC and the results are shown in Figure 5.7. The upper curves show the first and second heating and the melting temperature (Tm) of the crystalline phases of the polymer, while the lower curve refers to the cooling of the polymer after its first heating and the crystallization temperature (Tc). The second heating curve shows two peaks for the melting of the PCL, which is a semi-crystalline polymer [36]. The two peaks presented in Figure 5.7 (a) are associated with the melting of the pure PCL, showing that this polymer has two different crystallinity phases. For the first peak is possible to define a phase α with melting temperature at 42.3 °C and for the second peak is possible to define a phase β with melting

temperature at 48.1 °C. Similar results were obtained by Liu et al. [37] for PCL (Mw 2000), being the first peak at 45.1 °C and the second peak at 51.4 °C, both indicating the presence of two crystalline phases related to different degrees of organization or size in its crystallites.

Figure 5.7 – (a) Differential Scanning Calorimetry for PCL, (b) geranyl cinamate loaded PCL nanoparticles (1:3 geranyl cinnamate: PCL mass ratio) and (c) second heating heat flow rate for PCL and geranyl cinamate loaded PCL nanoparticles.





A compararison between the melting temperature of the pure PCL nanoparticles and of the PCL nanoparticles containing geranyl cinnamate shows a decrease of the smaller melting temperature from the 48.1 °C for 36 °C. When geranyl cinnamate was incorporated into the PCL nanoparticles, it was observed that the phase α and phase β maintain the same characteristic, but its melting temperature values decrease. By performing an analysis of the areas of the thermogrm's peaks a decrease of the enthalpy values of the both peaks (phase α and phase β) was observed for the PCL nanoparticles loaded with geranyl cinamate. The decrease of the enthalpie energy may indicate that the presence of the physically bonded between the ester molecules and the PCL polymer matrix generates a decrease of the degree of crystallinity of the nanoparticles. The enthalpy variation gives us data to establish that there was a 60.5% reduction in the degree of crystallinity of the PCL nanoparticles, indicating physical interections between geranyl cinamate and the PCL molecules, which hinders the crystallization of PCL nanoparticles. Due to the fact that the preparation of the nanoparticles involves the precipitation of the semicrystalline PCL in an aqueous environment it can occur that the polymer molecules rearrange, resulting in changes in crystallinity.

5.3.6 Encapsulation efficiency

The encapsulation efficiency (EE%) of the geranyl cinnamate in the PCL nanoparticles was evaluated using Equation 2 measuring the concentration of ester that was not encapsulated and remained in the aqueous phase. The EE% determined for three different formulations (FLN) with differents geranyl cinnamate:PCL mass ratios: 1:1 (FLN 1), 1:3 (FLN 2) and 1:10 (FLN 3), are presented in Table 5.2.

Table 5-2 - Encapsulation efficiency (EE%) for different geranyl cinnamate:PCL mass ratios determined using Equation 2.

Sample	geranyl cinnamate:PCL mass ratio	EE % ± S.D.
FNL 1	1:1	71.38 ± 1.60
FNL 2	1:3	95.68 ± 0.80
FNL 3	1:10	97.73 ± 1.30

A statistical analysis with $p < 0.05$ showed that the EE% increased when the concentration of geranyl cinnamate was reduced. The synthesis condition that favored the highest EE% was the dispersion FLN 3 with 1:10 of geranyl cinnamate:PCL. However, at this concentration the geranyl cinnamate loaded PCL nanoparticles is not able to inhibit the growth of bacteria according to microbiological tests employed to determine the MIC. Nevertheless, when higher amounts of geranyl cinnamate were encapsulated, the concentration was enough to the nanoparticles to exibit antibacterial action, according to MIC test.

Tampau, González-Martinez and Chiralt [38] encapsulated the carvacrol essential oil in PCL using miniemulsification/solvent evaporation method with an EE% of 80%, using a concentration of 15 wt.% of the essential oil in the organic phase. In addition, Pinto et al. [39] used PCL to encapsulate *Lippia sidoides* essential oil with EE% of 70.6%. In the work of Galindo-Perez et al. [40] an EE% of 85% was obtained for nanoparticles of PCL and β-carotene that were prepared by the miniemulsification/solvent evaporation method using Pluronic® F- 127 and sodium octenyl succinate starch (OSAstarch) as stabilizers. They also evaluated the EE% for Rosemary oil and Lemon oil, obtaining 81.53% and 92.29%, respectively.

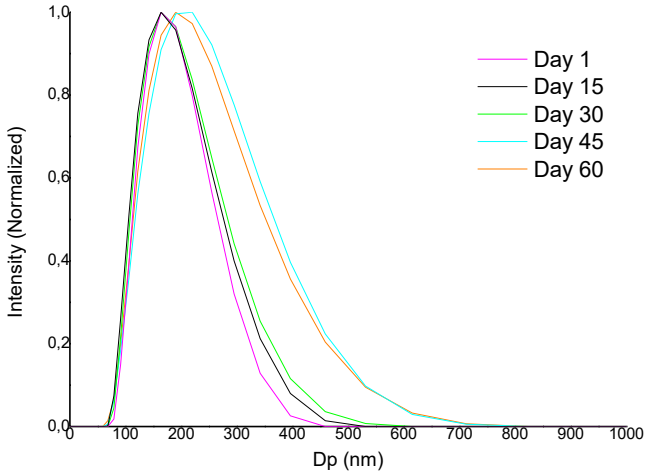
Sotelo-Boyas et al. [41] showed that PCL nanoparticles of thyme essential oil in lecithin were prepared by the solvent displacement technique and the EE% of thymol obtained was 68±1%.

5.3.7 Stability of geranyl cinnamate loaded PCL nanoparticles

The dispersions of geranyl cinnamate loaded PCL nanoparticles stored at 4 °C showed good stability over the course of 60 days. No significant visual modification was observed in the dispersions, such as change of color, precipitates or phase separation, therefore, all dispersions obtained with different geranyl cinnamate:PCL mass ratios could be classified as macroscopically colloidaly stable dispersions.

The mean nanoparticles diameter of the dispersions in different storage days was determined (186.8 ± 6.2 nm) and did not present any significant differences. Figure 5.8 shows the particle size distribution obtained by DLS for the nanoparticles dispersions with 1:3 geranyl cinnamate:PCL mass ratio. The results showed low values of polydispersity index (in the range of 0.1 to 0.2) and a non-significant variation of the mean size of the nanoparticles for all the suspensions stored over the days.

Figure 5.8 – Particle size distributions of PCL nanoparticles (1:3 geranyl cinnamate: PCL mass ratio) stored at 4ºC during 60 days.



These results show that the mean diameter of the nanoparticles are not modified over the days, suggesting absence of coalescence and degeneration. The Tukey test shows that the nanoparticles are stable when dispersed in aqueous media. There was no significant difference in the mean diameter of the suspended geranyl cinnamate nanoparticles stored

for 60 days, since the value found for $F_{calculated}$ = 0.0004246 was lower than the $F_{critical}$ = 2.4946, and also the P-value = 0.99, was greater than 0.05. The values obtained showed that there are not significant changes in the evolution of mean nanoparticles size values during the storage. This analysis was proven using a Tukey test in which data obtained for $F_{calculated}$ = 0.1615 being lower than the $F_{critical}$ = 3.8853, also for the P-value >0.05, the mean nanoparticles size distributions values showed no significant differences.

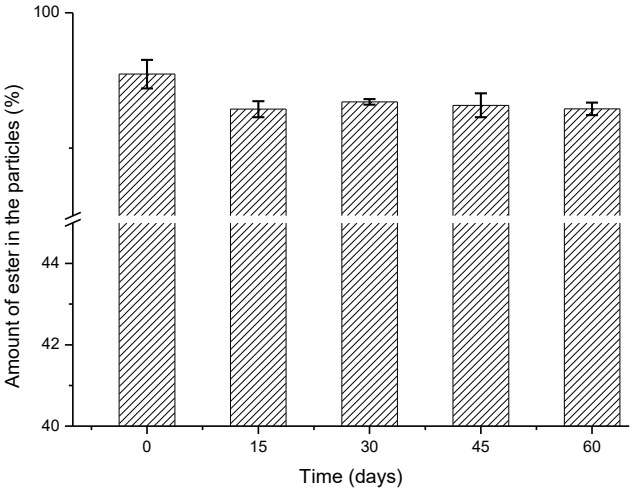
The average PdI value of 0.155 for the geranyl cinnamate loaded PCL nanoparticles indicated that a relatively narrow particle size distribution was obtained without aggregation or flocculation. Similar results were found by Galindo-Perez et al. [40], that obtained nanoparticles with Dp of 157.16 ± 2.95 nm and PdI of 0.151 ± 0.01 for curcumin encapsulated in PCL and nanoparticles with Dp of 245±13 nm (PdI of 0.099 ± 0.003) for the encapsulation of β-carotene in PCL using Pluronic® F-127 and sodium octenyl succinate starch (OSAstarch) stabilizers. Yegin et al. [41] prepared PCL nanoparticles loaded geraniol by nanoprecipitation technique and the Dp ranged from 26 to 412 nm.

The stability analysis of the nanoparticles was based on zeta potential (ζ) and revealed that the geranyl cinnamate loaded PCL nanoparticles dispersed in water, with pH 7.0, had low values of ζ, between -31 and -42 mV. Sodium dodecyl sulfate (SDS) is an anionic surfactant that when ionized in aqueous solution, provides negatively charged organic ions and is responsible for the negative ζ. Zeta potential is a physical property that is exhibited by the particles in aqueous dispersion and corresponds to the electrical potential of nanoparticles being affected by the nanoparticles surface composition and the medium in which they are dispersed [42]. This parameter is widely used to indicate stability in colloidal dispersions, where zeta potential values higher than 30 mV and lower than -30 mV promote high stability and prevent particles aggregation [43].

The pH of the dispersion affects the zeta potential and was monitored to evaluate the colloidal stability. A variation of the pH could indicate hydrolysis of the ester linkages of the PCL inducing the medium acidification due to the formation of carboxylic groups [25]. The values obtained showed that there are not significant changes in the evolution of pH values during the storage. This analysis was proven using a Tukey test in which data were obtained for $F_{calculated}$ = 0.6731 was lower than the $F_{critical}$ = 4.2564, also for the P > 0.05 the pH values showed no significant differences.

The ability to retain the geranyl cinnamate compound by the PCL nanoparticles during different time of storage in aqueous dispersion was also evaluated (Figure 5.9). For potential applications, it is very important that the nanoparticles are able to retain the active compound encapsulated. Statistical analysis showed that there was no significant difference in the EE% of geranyl cinnamate encapsulated in PCL particles during the storage time of 60 days, showing that the PCL nanoparticles had the ability of retain the active compound. The Tukey test showed a $F_{calculated}$ = 0.3866 smaller than the $F_{critical}$ = 3.8853; also a P = 0.6874 was obtained, greater than 0.05.

Figure 5.9 – Values of ester retention determined for geranyl cinnamate loaded PCL nanoparticles as a function of storage time in aqueous dispersions stored at 4ºC.

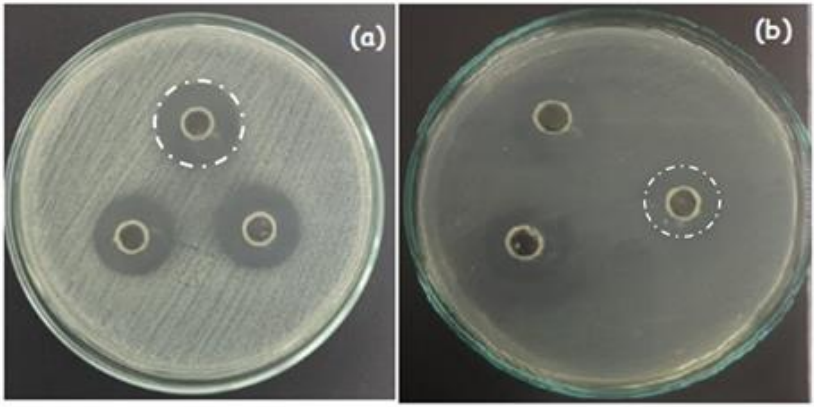


5.3.8 Microbiological tests

As a first analysis, the evaluation of the antimicrobial activity of pure geranyl cinnamate ester was carried out. Figure 5.10 shows the results of microbiological tests using agar diffusion for geranyl cinnamate synthesized in the optimum reaction conditions previously obtained and different types of bacteria. The geranyl cinnamate showed excellent antimicrobial activity for the two bacteria and a bactericidal effect. In the agar diffusion test for bacteria of type *Staphylococcus aureus* the

inhibition zone had an average diameter halo of 22.7±0.60 mm (Figure 5.10a). For *Escherichia coli*, the zone of inhibition had an average diameter of 17.2±0.32 mm (Figure 5.10b). These results corroborate with the results presented by Zanetti et al. [28].

Figure 5.10 - Agar diffusion test for geranyl cinnamate with different bacteria (a) *Staphylococcus aureus* and (b) *Escherichia coli*.



The methods establish that compounds with average diameters of halos of inhibition smaller than 9 mm should be considered microbiologically not active; average diameters between 9 and 4 mm microbiologically partly active; average diameters between 14 and 17 mm microbiologically active and average diameters higher than 17mm microbiologically very active.

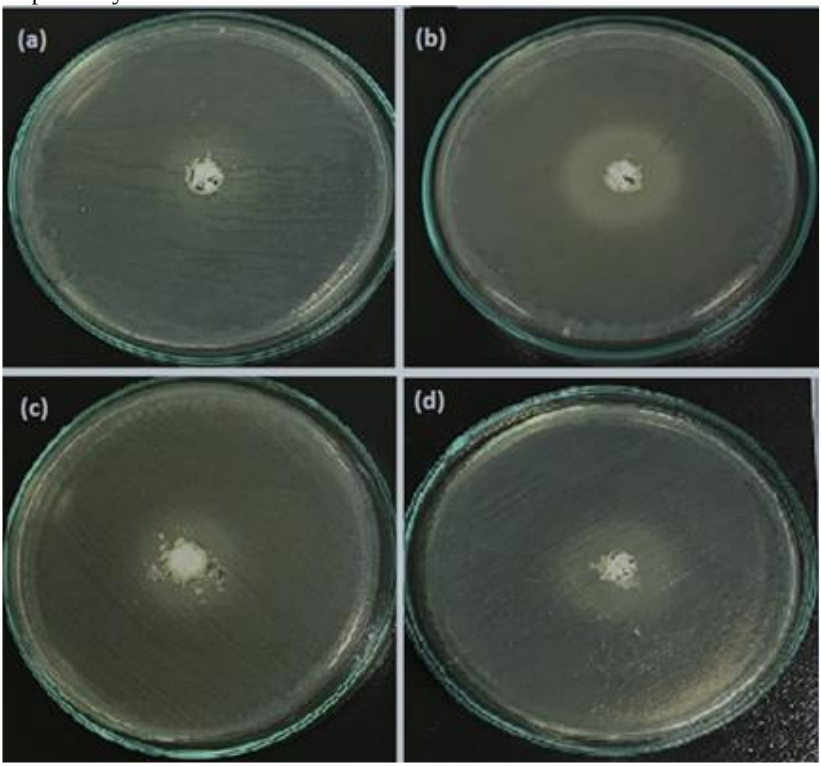
To evaluate the ability of the active compound to diffuse through the nanoparticle and inhibit microbial growth, an agar diffusion test was performed for the geranyl cinnamate loaded PCL nanoparticles against the same bacteria evaluated for the pure compound. Figure 11 shows the antibacterial activity of nanoparticles without geranyl cinnamate and nanoparticles with the geranyl cinnamate for the bacteria *Staphylococcus aureus* (Figures 5.11a and Figure 5.11b) and *Escherichia coli* (Figure 5.11c and Figure 5.11d).

By the agar diffusion tests there was no growth inhibition for the bacteria by geranyl cinnamate loaded PCL nanoparticles, showing no release of the active compound from the PCL nanoparticles. In order to evaluate if the active compound is able to leave the nanoparticles and inhibit the growth of bacteria, the minimum inhibitory concentration

(MIC) test was carried out, where the nanoparticles were dispersed in a solution containing water and 10% of DMSO, to a concentration of 5 mg mL^{-1} and then added in BHI medium with the same bacteria used on agar diffusion test. The nanoparticles remained on the MIC plate in contact with the bacteria for 24 hours.

In the MIC test it was possible to observe that the geranyl cinnamate did not diffuse out of the nanoparticles during the 24 hours exposure time, not inhibiting the growth of both bacteria tested. In order to prove the ability of PCL to trap the active compound inside the nanoparticles, a new test was performed, letting the nanoparticles in an aqueous solution with 10% DMSO during 12, 24, 48 and 72 hours. However, for all time intervals evaluated, diffusion and inhibition of bacterial growth did not occur.

Figure 5.11 – Microbiological evaluation of agar diffusion test for (a) and (b) PCL nanoparticles with *Staphylococcus aureus* and *Escherichia coli*, respectively; (c) and (d) geranyl cinnamate loaded PCL nanoparticles with 1:3 geranyl cinnamate: PCL mass ratio *Staphylococcus aureus* and *Escherichia coli*, respectively.



This result becomes positive, when the intention is to protect the active compound inside the nanoparticle. The release of the active compound can be induced according to the objective of the application of the nanoparticle.

5.4 **Conclusions**

Geranyl cinnamate loaded PCL nanoparticles were obtained with high encapsulation efficiency, mean size of 170 nm and a polydispersity index lower than 0.200. The aqueous dispersions of geranyl cinnamate loaded PCL nanoparticles stored at 4 ºC showed good colloidal stability over 60 days without the release of geranyl cinmnamate to the aqueous

phase. Microbiological results showed that geranyl cinnamate loaded PCL nanoparticles did not show antibacterial activity for Gram positive and negative bacteria as a results of the high hydrophobicity of geranyl cinnamate. These results suggest the need for the use of a trigger for the release of the active compound from the nanoparticle, which may be an oil phase or the use of an enzyme to degrade the polymer matrix, as lipase, depending on the application.

5.5 References

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6 SUPPLEMENTARY MATERIAL

In this chapter we have presented extra tests, but not yet published. For each of the items presented, the tests will be finalized, and the results will be organized and prepared in the form of articles for publication.

ITEM 1 - PCL AS A THERMAL PROTECTION OF THE ACTIVE COMPOUND

6.1 Nanoencapsulation of geranil cinnamate by policaprolactone as a thermal protection method

This chapter is based on the resume entitled "Nanoencapsulation of geranil cinnamate by policaprolactone as a thermal protection method" (abstract submitted and approved in ANM 2018), by Micheli Zanetti, Pâmela Fagundes, Thais Karoline Carniel, Alessandra Cristina Menezes, Débora de Oliveira, Pedro H. H. de Araújo and Márcio A. Fiori.

6.1.1 Introduction

Several studies of plant-derived antimicrobials have demonstrated the efficacy of these compounds when applied to food preservation, and have also investigated factors such as forms of exposure that influence the effectiveness of this protection[1]. In addition, the application of the essential oils in active packaging involves difficult operations because natural oils are very sensitive to thermal effects during the manufacture processes. One way to mitigate the negative effects of thermal and chemical decomposition is to encapsulate the essential oils in more resistant structures. Policaprolactone (PCL) is a compound that has been used for this purpose to encapsulate many organic compounds. Introduction[2]. The aim of this work was to show that from the nanoencapsulation of the geranyl cinnamate ester it is possible to obtain a natural antimicrobial compound with higher thermal stability and thus to potentiate it as a possible non-toxic antimicrobial additive for application in food packaging.

6.1.2 Experimental/theoretical study

The nanocapsules were obtained using PCL (PM 2000 $g.mol^{-1}$), dichloromethane CH_2CL_2 (PA 99.5%), geranyl cinnamate ester and Sodium Dodecyl Sulfate (SDS) as hydrophilic surfactant by means of the miniemulsion technique with solvent evaporation. The average diameter

(in intensity) and polydispersity index of the nanoparticles were determined using the Dynamic Light Scattering (DLS) technique. To analyze the thermal stability of the nanocapsules and its components was used Thermogravimetric Analysis (TGA), using a Schimadzu thermogravimetric analyzer (Model TGA 50) with a temperature range of 25 °C to 810 °C, a heating rate of 10 °C min-1 and under a nitrogen atmosphere with a flow rate of 50 $mL.min^{-1}$. The thermal stability evaluation of the nanocapsules was performed using samples with 0.02 g of capsules, which were submitted to the heat treatment in the greenhouse (Quimis brand) for 3 hours. The temperatures were defined according to the thermograms obtained by TGA, considering values higher than the volatilization temperature of the substrates, being 30, 60, and 90 °C. For this analysis the concentration of the ester present in the capsules, with and without heat treatment, was measured by spectrophotometer at the wavelength of the compound which is 273 nm.

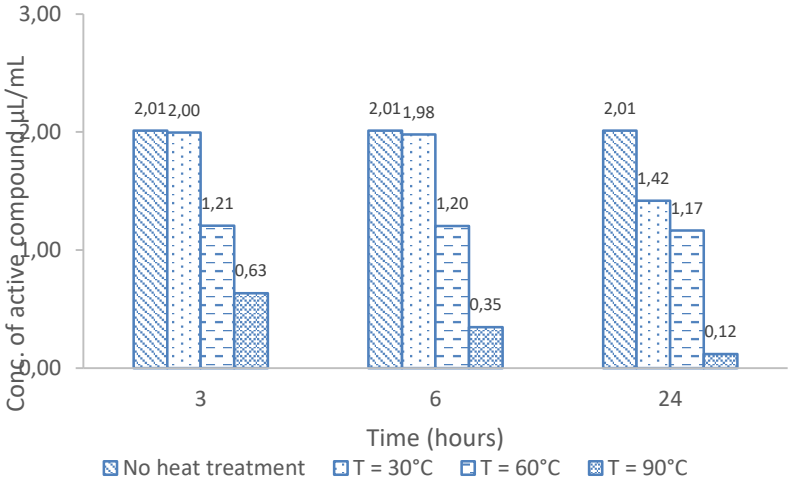
6.1.3 Results and discussion

The PCL nanocapsules without geranyl cinnamate ester had an average size of 80.70 nm and a polydispersion index of 0.172. The PCL nanocapsules synthesized with geranyl cinnamate ester presented an average size of 87.86 nm and a polydispersion index of 0.163.

These values are small and may be associated with a small variation in the size of and also the presence of a homogeneous product and without aggregation or flocculation of the formulations.

Figure 6.1 shows the comparasion between the concentration of geranyl cinnamate molecules in the capsules before and after the heat treatment. The results show that a significant fraction of ester is released during the heat treatment at temperatures above the PCL melting temperature.

Figure 6.1 - Concentration of geranyl cinnamate molecules in the capsules before and after the heat treatment.



The results show that more than 90% of the encapsulated ester molecules are released after the heat treatment at temperatures of 90 °C. The results of the thermal analysis of the compounds (Thermogravimetric Analysis - TGA and Differential Scanning Calorimetry - DSC) show that pure oil undergoes volatilization at a temperature of 40ºC. With the encapsulation these analyzes show that the thermal stability of the encapsulated compound rose to 60 °C. At this temperature, the PCL starts to melt and consequently the active compound is released. However, only for temperatures greater than 80 °C is there significant release of the active compound.

These results are very important because they prove that the temperature can be a trigger of release of the active compounds.

6.1.4 Conclusion

The results obtained show that PCL capsules were obtained on nanometric scale. The encapsulation process allowed to protect the substrates from degradation effects. The encapsulated geranyl cinnamate ester was not released at lower temperatures. After the heat treatment.

ITEM 2 – ANTIFUNGIC ACTIVITY EVALUATION TEST

6.2 Evaluation of the antifungal activity geranyl cinnamate ester

Tests were performed to evaluate the ability of the geranyl cinnamate ester to act as an antifungal compound. The procedures adopted, as well as the results obtained are presented below. The results obtained will be published as a short comunication.

6.2.1 Antifungal characterization of the geranyl cinnamate ester

The geranyl cinnamate ester was submitted to the evaluation of antifungal activity with three types of fungi: yeast *Candida albicans* (ATCC 24433), *Penicillium variabile* (ATCC 32333) and *Aspergillus niger* (ATCC 16888).

The strains used of Candida albicans were obtained from the Laboratory of Mycology of the Community University of the Region of Chapecó - Unochapecó. The strains were reactivated in Sabouraud-Dextrose Broth and for conduction of the study fungal suspensions of the microorganisms were prepared by choosing five colonies with a diameter of approximately 1 mm after incubation of 24 h of the Candida species. The colonies were suspended in 5 mL of sterile saline (0.85% saline) and the resulting suspension was homogenized on a vortex shaker for 15 seconds. Subsequently, sufficient saline was added to obtain equivalent turbidity of a standard solution of the McFarland 0.5 scale, so as to obtain a standard yeast suspension containing approximately 10^6 microorganisms ml^{-1}.

Solutions with different concentrations, as presented in Table 6.1, were prepared by diluting geranyl cinnamate in a solution containing 90% sterile water and 10% DMSO. The antifungal activity was evaluated by the solid medium diffusion method in Petri dishes containing Sabouraud Dextrose Agar with Chloramphenicol, where 6 mm diameter filter paper disks were impregnated with 10 µL of each prepared geranyl cinnamate solution previously. The discs were then placed on the plates, previously inoculated with 100 µL of fungal suspension.

The system was incubated at 36 °C for 48 h in a bacteriological oven and then the inhibition halo readings were performed. The zones of growth inhibition were measured with pachymeter, previously calibrated.

The whole experiment was performed in triplicate. It was considered inhibition halo equal to or above 8 mm in diameter, according to the criteria of Parekh and Chanda (2007). For the viability control of the standard Candida albicans strain, a solution containing only the 90% solution of sterile water and 10% of DMSO, called the control solution, was used.

The evaluation of the antifungal activity of the geranyl cinnamate ester against the *Aspergillus niger* and *Penicillium variable* fungi was based on the in vitro bionithic method, by means of which the effects of the geranyl cinnamate ester in different concentrations (as in Table 6.1) were evaluated mycelial growth of the fungal culture, by means of comparison with the control.

Lyophilized fungal ATCC strain was hydrated in BHI broth for 48 hours in an oven at 28 °C. After this time, the BHI showed fungal activity, becoming cloudy, then the 10μL volume of the BHI fungus was removed and seeded in Petri dishes containing Sabouraud Dextrose Agar with Chloramphenicol. For the research with *Aspergillus* and *Penicillium*, the peal was carried out from the culture of the fungus in the medium Sabouraud Dextrose Agar with Chloramphenicol, by means of the scraping method, with handle in μL. The geranyl cinnamate solutions at the different concentrations were deposited on the culture medium before the peal in a quantity of 100 μL on each plate and were spread on the medium with the aid of the sterile Drigalski loop. The reading for plates containing *Aspergillus niger* and *Penicillium variabile* is always performed in 7 days after incubation at 28-30 °C.

The percent inhibition of mycelial growth was calculated using Equation 2 (Lindsey & Standen, 2004):

$$\%_{inhibition} = 100 - \left(\frac{E}{C} * 100\right) \qquad \text{Eq. 2}$$

At where:

E = mean of mycelial growth of each treatment; and C = mean control mycelial growth (control).

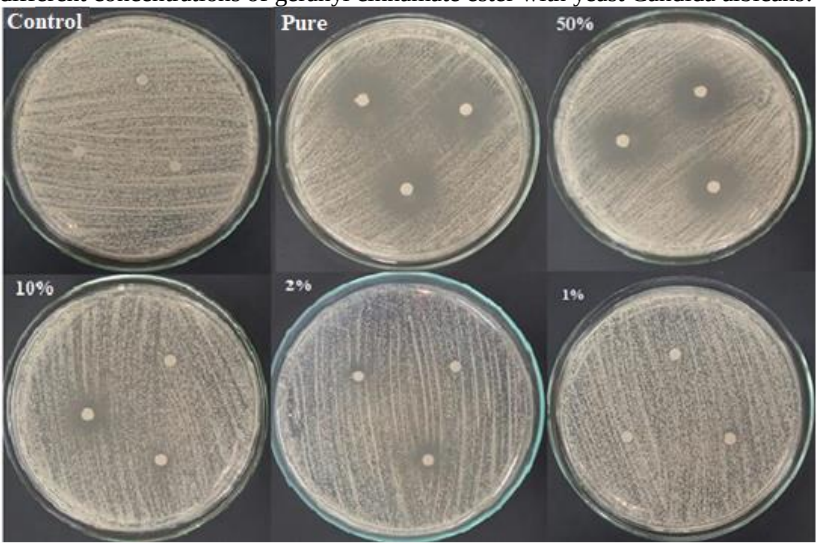
Table 6-1 – Dilutions of the geranyl cinnamate ester applied in Petri dishes for antifungal tests.

Samples	Dilution (µL of ester / µL of Water/DMSO solution)	Concentration (µL of ester / mL of Water/DMSO solution)
Control	0	0
Pure	-	-
50%	100/100	1000
10%	100/1000	100
2%	100/5000	20
1%	100/10000	10

6.2.2 Results obtained for the antifungal activity of the geranyl cinnamate ester

The first evaluation of the antifungal activity of the geranyl cinnamate ester was carried out with yeast *Candida albicans*. Tests were performed with five different concentrations of the geranyl cinnamate ester and the diameters of the growth inhibitory halos of *Candida albicans* were compared to the control sample, which contained only a solution of 90% water and 10% DMSO. Figure 6.2 shows the results obtained with agar diffusion tests.

Figure 6.2 - Results of the agar diffusion assays for the antifungal activity of different concentrations of geranyl cinnamate ester with yeast Candida albicans.



The geranyl cinnamate ester was shown to be very active against yeast Candida albicans when used neat, with mean halo diameter value of 25.30 ± 1.10 mm, and up to concentration of 1000 μL mL^{-1} mean halo diameter values of 24.00 ± 0.60 mm. At concentrations greater than 100 μL mL^{-1} the halos formed have disc order diameters and therefore the geranyl cinnamate ester is not active. At a concentration of 10 μL mL^{-1} the ester showed no antifungal activity. It is possible to observe that in the control sample there was no inhibition of growth, which proves that the solution used in the ester dilution does not affect the growth of fungi.

In a comparative study of the antimicrobial activity of an essential oil containing in its composition thymol, carvacrol and geraniol against the fungus *Candida albicans*, Botelho et al. (2007) demonstrated that the three compounds exhibited antifungal activity. Also in the work of Marcos-Arias et al. (2011), the authors report that geraniol showed antifungal activity against strains of *Candida albicans.*

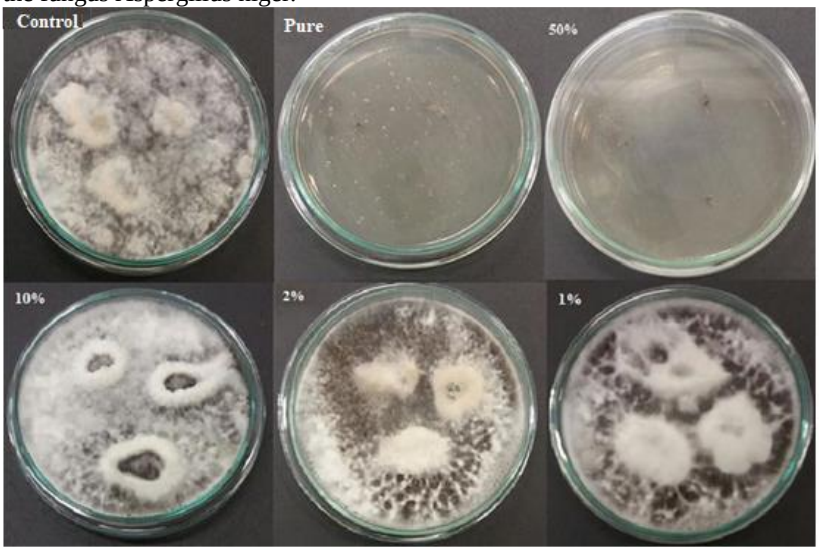
The antimicrobial activity of essential oils in bacteria and fungi occurs similarly. The constituents of the oils destroy the cytoplasmic membrane and the cell wall of bacteria and fungi. This effect results in the extravasation of the cytoplasm and its coagulation, in addition to inhibiting cellular respiration (Cox et al., 2000).

The antifungal activity of the geranyl cinnamate ester was also evaluated with the fungus *Aspergillus niger*. The assays were based on the in vitro bioanalytical method (Lindsey and Standen, 2004), which evaluates the mycelial growth of the fungal culture by comparison with the control, which is performed with a solution of 90% water and 10% DMSO and without the active principle. The tests were performed with different concentrations of geranyl cinnamate ester, and the results are shown in Figure 6.3.

The geranyl cinnamate ester showed antifungal activity against *Aspergillus niger* fungus when used with a concentration of up to 1000 μL mL^{-1}. In the scientific literature there were no studies related to the geranyl cinnamate ester, only some relating the geraniol activity, but without the optimization of the concentrations in the synthesis reactions.

The evaluation of the antifungal activity of the geranyl cinnamate ester with the fungus Penicillium variable followed the same procedure used with *Aspergillus niger*. The results are shown in Figure 6.3.

Figure 6.3 – Results of antifungal tests by diffusion in agar for different concentrations of geranyl cinnamate ester diluted in 10% DMSO solution with the fungus Aspergillus niger.

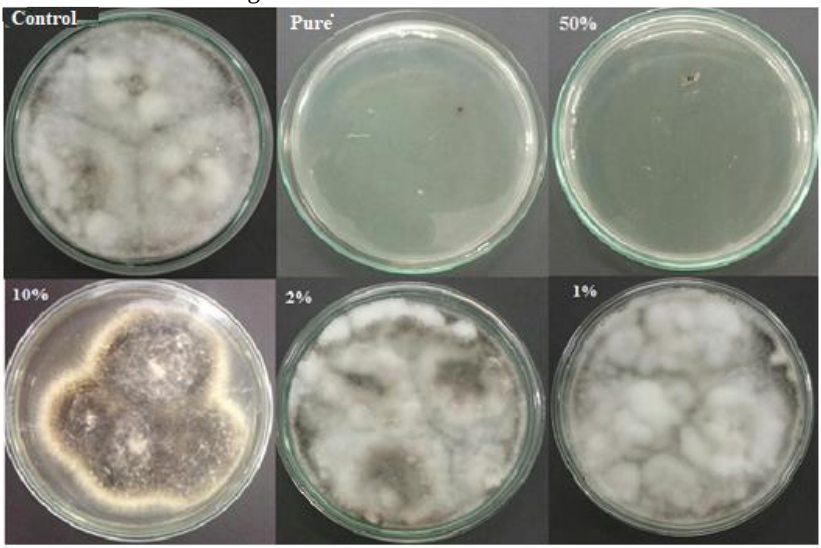


The geranyl cinnamate ester demonstrated antifungal activity against the fungus Penicillium variable when used with concentrations of 1000 μL mL^{-1}, being the inhibition of 100% of the analyzed area. For the

concentration of 100 μL mL^{-1} the antifungal activity was 16%. For the other concentrations, the ester showed no antifungal activity.

In the work of Dantas (2013), the significant fungicidal effect of thymol on *Penicillium* strains was observed, where a total inhibition of the mycelial growth of the strains was observed in three concentrations tested (512, 256 and 128 μg mL^{-1}). Franco and Bettiol (2002) presented results indicating that some essential oils like oil of *Mentha arvensis* (mint), *Cymbopogon citratus* (citronella), *Eucalyptus* spp. (Eucalyptus), *Rosmarinus officinalis* (rosemary), *Zingiber officinale* (ginger), present antimicrobial properties capable of controlling *Penicillium digitatum* in orange fruits, and may be an alternative to the use of synthetic fungicides.

Figure 6.4 – Results of antifungal tests by diffusion in agar for different concentrations of geranyl cinamate diluted in 10% DMSO solution with the Penicillium variable fungus.



Therefore, the data obtained in this stage of the work confirm the antimicrobial activity of the geranyl cinnamate ester with bacteria and fungi tested, showing the importance that this compound may have in an area where few studies are published for growth control, mainly fungi, using natural compounds.

ITEM 3 – INVESTIGATION OF ACUTE TOXICITY

6.3 Investigation of Geranyl Cinnamate Acute Toxicity

The experiment was conducted in accordance to the recommendation of the OECD Guideline 423 (OECD, 2001).

Female Swiss mice (32–36 g, n=3, 8-12 weeks, nulliparous and non-pregnant) bred in-house were used in all experiments. Animals were kept in a controlled environment (22 ± 2°C) with a 12-h light/dark cycle (lights on 6:00 a.m. to 6 p.m.) and fed standard lab chow and water ad libitum. Animal care and experiments were conducted in accordance to Brazilian law (Brazil, 2008; 2017) and European Communities Council Directive of 24 November 1986 (86/609/EEC). All experiments were carried out between 8:00 a.m. and 6:00 p.m.

The geranyl cinnamate was administered in a single dose by gavage. The dose of 300 mg/Kg (p.o.) was selected due to the lack of information of toxicity regarding the geranyl cinnamate. The animals were fasted before geranyl cinnamate administration (food but not water was withheld for 3.5h) and observed during the 4 h after treating, and every day for 14 days. Toxicity signs and symptoms considered were piloerection, muscular tonus, abdominal writhing, palpebral ptosis, motor activity, hypothermia, shacking, posterior paws paralysis, salivation, bronchial secretion, diarrhoea, lethargy, convulsions and death. Mice body weight and food consumption were registered across the whole period.

The geranyl cinnamate was dissolved in corn oil (OECD, 2001), due to its low solubility in water. The lack of corn oil toxicity has already been demonstrated (Coldham et al., 1997).

Food intake as well as animals' weight were analyzed by using two-way repeated measures ANOVA. $P < 0.05$ was considered as significant for all analyses. Data are expressed as mean ± S.E.M.

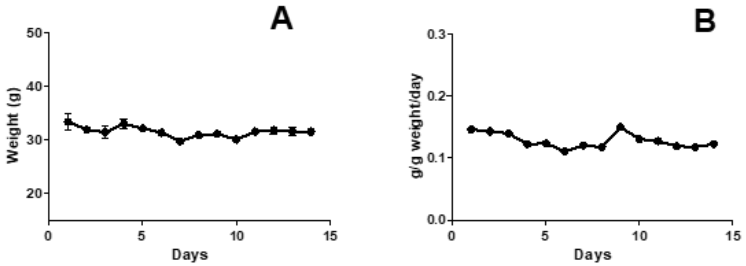
6.3.1 Results

The general behavioral of the geranyl cinnamate-treated animals (300 mg/kg, p.o.) was observed first for a short period (4 h) followed by a long period (14 days). Mice did not display any drug related changes in behavior, except that palpebral ptosis was after 3 h after the oral administration. No mortality was observed after oral administration of the tested compound (Table 6.1). Mice body weight as well as food consumption were not affected by acute oral administration of geranyl cinnamate 300 mg/kg, p.o. (Fig. 6.5).

Table 6-2 - General appearance and behavioral observations of acute toxicity study (geranyl cinnamate acutelly administered at 300 mg/kg, p.o.).

Toxicity sign	Geranyl cinnamate (300 mg/kg, p.o.)
Piloerection	No effect
Muscular tônus	No effect
Abdominal writhing	No effect
Palpebral ptosis	Observed 3 h after the first administration
Motor activity	No effect
Hypothermia	No effect
Shacking	No effect
Posterior paws paralysis	No effect
Salivation	No effect
Bronchial secretion	No effect
Lethargy	No effect
Diarrhoea	No effect
Convultions	No effect
Death	No effect

Figure 6.5 - Mice body weight (g) (panel A) and food intake (g/g weight/day, panel B) after geranyl cinnamate (300 mg/kg, p.o.) acute administration. Two-way repeated measure ANOVA. Data are expressed as mean ± S.E.M.



6.3.2 Conclusion

In conclusion, according to the OECD (2001) acute toxicity parameters, the geranyl cinnamate did not induce toxicity to mice, at 300 mg/kg, by oral route. These results indicate that this compound might be tested at the limit dose (2000 mg/kg, p.o.), carried out with six animals (three animals per step). Considering the OECD guideline 423, we may

infer that the geranyl cinnamate LD50 should be between 300 – 2000 mg/kg.

7 CONCLUDING REMARKS

Like any other thesis, this document represents a very small part of a very broad study, and much has yet to be explored. However, the information obtained in this work may contribute to an advance in the transformation of theoretical studies into commercial products.

Basically, this thesis aimed to provide contributions to the field of nanoencapsulation of natural antimicrobial compounds. Relevant information was obtained in this work, the main ones being:

- The essential oil of geraniol showed excellent antimicrobial activity against the tested bacteria, as well as bactericidal effect. In the agar diffusion test geraniol was shown to be a microbiologically very active compound for the bacteria *Staphylococcus aureus*, *Escherichia coli, Salmonella enterica* and microbiologically active for the bacterium Listeria monocytogenes.

- By an enzymatic catalysis esterification route with immobilized *Candida antarctica* NS88011, it was possible to obtain high ester conversions (97%) under the reaction conditions of 80 °C, 20% by weight of *C. antarctica*, and for molar ratio of geraniol and cinnamic acid of 5: 1. The antimicrobial activity of the geranyl cinnamate ester was tested on the bacteria *Staphylococcus aureus* and *Escherichia coli* and three types of fungi: yeast *Candida albicans*, *Penicillium variabile* and *Aspergillus niger*, and by the agar diffusion test the compound showed an excellent antimicrobial activity for all the microorganisms tested.

- It was possible to obtain nanocapsules loaded with geranyl cinamate with polycaprolactone polymer. The characterization results showed that nanocapsules with manometric sizes in the 170 nm range and with a polydispersity index of less than 0.200 were obtained, showing the formation of homogeneous products in relation to the particle sizes and without the aggregation or flocculation of the formulations.

- Stability tests showed through the analysis of the pH of the synthetic solutions, the average particle size, the polydispersity index, the zeta potential and the encapsulation efficiency, that the suspensions of geranyl cinnamate ester nanocapsules, stored at 4 ºC presented good stability for 60 days, with no significant change in the items analyzed.

- It has also been shown that nanoencapsulation as PCL increases the thermal stability of the geranyl cinnamate ester. The thesis that the polycaprolactone -PCL is able to provide protection to the active compound can be proved by the results of TGA, which presented good indications that the encapsulation of the geranyl cinnamate ester by PCL occurs, and the encapsulation increased the temperature for the release of the geranyl cinnamate ester at 20 ° C. In the standard of FTIR analysis of geranyl cinnamate nanocapsules, it was possible to observe the prevalence of the characteristic bands of the pure ester and the pure polymer, confirming the presence of these bands in the nanocapsules.
- Microbiological tests showed that the geranyl cinnamate ester did not diffuse from the nanocapsules during the maximum exposure time of 72 hours in solution (90% water and 10% DMSO) and did not inhibit the growth of both bacteria tested. This result becomes positive when the intention is to protect the active compound within the nanocapsule. The release of the active compound can be induced according to the purpose of the application of the nanocapsules.

- A possible trigger test for release of the geranyl cinnamate ester from within the capsule was through the use of temperature, as presented in the chapter The Plus of Thesis. The use of the capsules in processes involving temperatures above 60 °C, causes the nanocapsule to rupture and the release of the active compound. If the aim is to use nanocapsules with the active compound in obtaining a polyethylene polymer film, for example, the film processing temperature may be the trigger for the release of the compound from the capsule and impregnation thereof in the film, making the packaging microbiologically active.

- Thus, it was possible to conclude that stable nanocapsules capable of protecting a natural active compound were obtained in this work. Future works may potentiate these nanocapsules for a possible non-toxic antimicrobial additive for application in food packaging.

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