Chitosan Film with *Citrus limonia* Essential Oil: Physical and Morphological Properties and Antibacterial Activity

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**Abstract:** The development of active packaging for food preservation is attracting increased attention due to serious environmental problems caused by synthetic and conventional materials. In the present study, the physical, chemical, optical, microstructural, and antibacterial properties of chitosan films with *Citrus limonia* essential oil (CEO) were investigated. The incorporation >0.75% of CEO increased the thickness of the films. The incorporation >0.25% of CEO reduced the moisture content and the water vapor permeability of the chitosan films. The biodegradability of the films over ten days ranged from 55.46–62.65% and was not affected by the addition of CEO. All films showed good UV light barrier properties, and the incorporation of the CEO caused a decrease in the visible light transmission rate values. The addition of CEO changed the color of the bioactive films significantly, remaining darker and yellowish. The bioactive films showed antibacterial activity against *Staphylococcus aureus*, but not against *Escherichia coli*. The films showed a heterogeneous microstructure with oil droplets retained in the continuous polysaccharide network. The results showed that chitosan films with CEO are promising as an active packaging material for food preservation.

**Keywords:** active packaging; lime; biofilm

1. Introduction

Petrochemical-based polymers are commonly used in food packaging due to their low cost and easy manufacturing. However, these materials have introduced environmental problems from plastic waste due to their exceptionally long degradation time in nature. Bioplastic materials—defined as biodegradable plastic with bio-based content or both—appear as alternatives to replace conventional plastics, due to the minimization of environmental problems associated with the use of plastics derived from petrochemicals. These materials may be part of the solution in the fight against climate change [1–3]. As an alternative approach to petrochemical-based polymers, natural biopolymers have increasingly attracted attention to the development of biodegradable and active food packaging [4–6].

Biodegradable films are produced using macromolecules such as polysaccharides, proteins, lipids or a combination of these compounds [7]. Among these macromolecules, chitosan is a natural polysaccharide formed from the repetition of beta (1–4) 2-amino-2-deoxy-d-glucose (or d-glucosamine) units, presenting a polymeric chain similar to that of cellulose (except for replacing the hydroxyl
groups in position 2 with acetamido groups) [5]. As a polysaccharide, chitosan was designed as a film material due to its good film-forming physical, antimicrobial and antioxidant properties [8–10].

Biodegradable and active packaging is an innovative packaging approach that can protect food and extend shelf life due to its ability to carry antioxidant and antimicrobial activities, among other active ingredients [11,12]. In this sense, the incorporation of essential oils (EOs), to improve the antimicrobial and antioxidant activity of films, has been explored for being natural, safe, biodegradable alternatives, and for presenting higher antimicrobial and antioxidant activity [4,13,14].

EOs are complex mixtures of volatile organic substances, consisting of oxygenated compounds and hydrocarbons, such as sesquiterpenes and monoterpenes, produced by aromatic plants as secondary metabolites [15,16]. These compounds are responsible for the antimicrobial activity of EOs, and they have mechanisms associated with their lipophilic nature, which interacts with microbial membranes leading to leakage of cell compounds and causing energy losses from microbial cells [17,18].

The species Citrus limonia Osbeck, originally from India, is known as “china lime”, “pink lime”, or “vinegar lime”. This species is grown in orchards and can be used as a source to obtain EOs with bioactive potential [19,20]. Pires & Piccoli [19] demonstrated that C. limonia EOs showed antifungal activity against Candida utilis and antibacterial activity against Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa. Subsequently, Estevam et al. [20] reported that Citrus limonia EOs showed antibacterial activity against Streptococcus mitis (ATCC 49456), Streptococcus mutans (ATCC 25175), Streptococcus sanguinis (ATCC 10556), Streptococcus sobrinus (ATCC 33478), and Bacteroides fragilis (ATCC 25285).

Although it seems to be an attractive active agent, to our knowledge, there are no reports in the literature on the incorporation of C. limonia EOs as an active agent in biopolymeric films. In this context, the objective was to develop and characterize biodegradable films of chitosan incorporated with the essential oil extracted from C. limonia leaves.

2. Materials and Methods

2.1. Materials

The bacterial strains Staphylococcus aureus ATCC 25923 and Escherichia coli O157:H7 were obtained from the Microbiology Laboratory of the Brazilia Federal Institute of Education, Science and Technology, Campus Planaltina. Chitosan (viscosity 20–300 cP, deacetylation degree 95–98% and medium molecular weight of 90–310 kDa) and glycerol were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). All other solvents and chemicals used were of analytical grade.

2.2. Essential Oil (EO) Extraction

Fresh leaves of C. limonia were collected in the region of Rio Verde-GO in August 2015 and a sample was deposited at the Herbário Jataiense Professor Germano Guarin Neto under registration No. HJ 7522. The fresh leaves were reduced in a knife mill and subjected to extractions by hydrodistillation method in a Clevenger-type apparatus for 2 h from boiling [20]. The EO extracted was packed in amber glass bottles and kept refrigerated at 4 ± 2 °C, until it was incorporated into the filmogenic solutions.

The chemical characterization of the C. limonia OE (Table 1) was previously carried out and published by our research group [20]. Using gas chromatography and mass spectrometry (GC–MS, QP-5000, Shimatzu, Columbia, MD, USA), 18 chemical constituents were identified for C. limonia EO (total of 91.9%), including limonene (1) (46.3%), nerol (2) (10.0%), and 1,8-cineole (3) (13.4%) (Table 1).

2.3. Preparation of Films

Chitosan-based films were produced according to the method described by Ojagh et al. [21] with some modifications. The chitosan-based film was prepared by dissolving chitosan in an aqueous solution (1% v/v) of glacial acetic acid to a concentration of 2% (w/v) while stirring on a magnetic stirrer/hot plate. The solution was mixed with low heat (at 40 °C), which typically required 6 h stirring,
The resultant chitosan solution was filtered through a Whatman No. 3 filter paper to remove any undissolved particles. After filtration, the solution was returned to the magnetic stirrer/hot plate and glycerol was added as a plasticizer to a level of 0.75 mL/g chitosan. The plasticizer was mixed into the solution for 30 min. Then Tween 80 was added as an emulsifier at the level of 0.2% (v/v) of EO to assist essential oil dissolution in film-forming solutions. After 1 h of stirring, EO was added to chitosan solution to reach a final concentrations of 0.4%, 0.8%, 1.5%, and 2% (v/v) as essential oil concentration per film in emulsifying equipment (TE-139, Tecnal, Piracicaba, Brazil) at 900 rpm for 2 min. After cooling to room temperature, the film-forming solution was degassed under vacuum for 5 min. The film-forming solutions (160 mL) were cast on the center of 27 × 27 cm² glass plates and then dried for 30 h at ambient conditions (25 °C). Dried films were peeled and stored in a desiccator at 25 °C and 51% relative humidity until evaluation. Saturated magnesium nitrate solution was used to meet the required relative humidity.

### Table 1. Chemical composition of *Citrus limonia* OE using GC–MS [20].

| Compounds                | RI Literature | Calculated | Area (%) |
|--------------------------|---------------|------------|----------|
| Myrcene                  | 990           | 977        | 0.8      |
| Limonene                 | 1029          | 1028       | 40.0     |
| Nerol                    | 1361          | 13.67      | 6.8      |
| 1,8-Cineol               | 1031          | 1030       | 13.4     |
| (trans)-Limonene oxide   | 1142          | 1137       | 3.0      |
| Caryophyllene oxide      | 1583          | 1586       | 6.9      |
| Geraniol                 | 1267          | 1274       | 3.4      |
| Neral                    | 1238          | 1243       | 3.9      |
| Linalool                 | 1096          | 1100       | 2.9      |
| (cis)-Linalool oxide (furan) | 1072      | 1073       | 1.2      |
| p-Cymene                 | 1024          | 1024       | 0.7      |
| Sabinene                 | 1070          | 1067       | 0.3      |
| Isopulegol               | 1159          | 1156       | 1.7      |
| Pinocarvone              | 1164          | 1163       | 0.7      |
| α-Terpineol              | 1118          | 1192       | 2.3      |
| (trans)-Carveol          | 1216          | 1220       | 1.7      |
| (4E)-Decen-1-ol          | 1262          | 1260       | 1.0      |
| Citronellal              | 1273          | 1276       | 1.2      |
| Total (%)                | -             | -          | 91.9     |

### 2.4. Bioactive Film Characterization

Film thickness was measured with a manual micrometer (0.01 mm precision) in ten repetitions and at random positions. Film water content was measured using 2-cm² film samples of known mass and dried at 105 °C for 24 h.

The film degradation test (biodegradability) was performed according to the methodology described by Stoll et al. [22] with modifications. A natural organic soil contained in plastic boxes was used as the degradation environment. The film samples were cut into rectangles (2 cm × 3 cm), dried at 60 °C to constant weight (w0), placed in a plastic mesh, and buried to a depth of 5 cm below the soil surface. Every other day, the soil was watered to maintain moisture at approximately 40%. The rate of degradation of the films was determined after 10 days as weight loss (w10) calculated \((w_{10} - w_0)/m_0\) × 100 and expressed in percentage (%).

The water vapor permeability (WVP) of the film was measured as described by Casariego, Souza and Cerqueira [23]. The films were sealed in permeation cells containing 3 g of calcium sulfate to simulate the 0% RH storage condition. Cells were initially weighed and placed in a desiccator containing saturated potassium sulfate solution (97% RH at 25 °C). Film weight gain of permeation cells was determined at 2-h intervals over 10 h. The water vapor transmission rate (WVTR) (g/m² h) was determined from the slope of the regression analysis of the water weight gain (Δa) transferred...
through a film area (A) for a defined time (Δt) (Equation (1)). The WVTR of the films was used to calculate the water vapor permeability coefficient (WVP) using Equation (2).

\[
\text{WVTR} = \frac{\Delta a}{A \Delta t} \quad (1)
\]

\[
\text{WVP} = \frac{\text{WVTR}}{\Delta p} \times x \quad (2)
\]

where WVP is the permeability coefficient (g mm/m² h Pa), x is the film thickness (mm), A is the film area exposed (19.625 × 10⁻⁶ m²), and Δp is the partial water vapor pressure gradient between the inner (p1) and outer (p2) surfaces of the film in the chamber (Δp = 307,93 Pa at 25 °C).

The solubility in water of the films was determined as described by Kavoosi et al. [24], with modifications. The initial dry mass was determined with 2 cm² film samples cut and dried at 100 ± 5 °C for 24 h. The samples were soaked in 50 mL of distilled water and after 24 h at 23 ± 2 °C, they were dried again at 100 ± 5 °C for 24 h to obtain the final dry mass. Film water solubility was calculated using Equation (3).

\[
\text{Solubility (\%)} = \frac{\text{initial dry mass} - \text{final dry mass}}{\text{initial dry mass}} \times 100 \quad (3)
\]

The color analysis of the films was determined using a Color Quest II spectrophotometer (Hunter lab, Reston, VA, USA) using the CIEL*a*b* system, and the chroma and hue values were calculated based on the parameters, a* and b*.

The film barrier properties against visible light were measured at 250–800 nm using a UV-Vis spectrophotometer (Spectrometer Lambda 750, PerkinElmer, Shelton, CT, USA).

The microstructural analysis of films with increments of 1000x was conducted using an electronic scanning electron microscope (acceleration voltage 2.5 kV, JSM 6610, JEOL, São Paulo, Brazil) equipped with EDS (NSS Spectral Imaging, Thermo Scientific, Waltham, MA, USA) utilizing degreased and dried samples.

2.5. Antibacterial Activity of the Bioactive Films

Antimicrobial activity was evaluated in vitro against two bacteria (Staphylococcus aureus and Escherichia coli). Briefly, 200 µL of bacterial culture (adjusted to 10⁴ cells/mL) were grown on plates with tryptone agar medium and 200 µL of spore suspension (adjusted to 10⁵ spores/mL) on plates with plant count agar (PCA). The films (10-mm diameter) were then placed on the surface of the agar and incubated at 37 °C for 24 h. The diameter of the zone of inhibition was measured with a pachymeter.

2.6. Statistical Analysis

The experiment was replicated three times. In each replication, analyses were conducted in triplicate. The results correspond to the mean ± standard deviation of the mean. Data were analyzed by one-way analysis of variance (ANOVA) whereas Tukey’s test (p < 0.05) was used for testing differences between the means using Assistat software version 7.7 (Professor Francisco de A. S. e Silva, Federal University of Campina Grande, Brazil).

3. Results and Discussion

In the present work, the films developed were characterized and the results were compared with those obtained for the film without EO (chitosan film) and with results reported in the literature since the incorporation of active agents can influence their properties. All films were homogeneous, flexible, with no brittle areas and no bubbles.

Table 2 shows the physical properties of the chitosan films incorporated with C. limonia EO (CEO). As expected, the incorporation of CEO increased significantly (p = 0.034) the thickness of the
chitosan films from the concentration of 0.75%, which can be attributed to the increase in the number of molecules with the increased inclusion of essential oil, causing an increase in the free volume of the film. This behavior was enhanced when *Rosmarinus officinalis* L., *Artemisia herba alba* Asso, *Ocimum basilicum* L., and *Mentha pulegium* L. EOs were incorporated in sodium alginate films [4], as well as when clove EO was incorporated into pectin films [25].

![Image of page content]

**Table 2.** Physical properties of chitosan films with *Citrus limonia* essential oil.

| Film     | Thickness (mm) | Moisture (g/100 g) | Solubility (%) | Water Vapor Permeability (g/m²/day) | Biodegradability (10 days, %) |
|----------|----------------|--------------------|----------------|------------------------------------|-------------------------------|
| Control  | 0.17 ± 0.04 b  | 24.80 ± 0.34 a     | 46.65 ± 2.49 a | 341.64 ± 9.35 a                     | 62.65 ± 1.03 a                |
| 0.25EO   | 0.18 ± 0.03 b  | 23.00 ± 0.65 b,c   | 38.78 ± 0.55 b | 320.07 ± 6.52 b                     | 55.46 ± 0.90 a                |
| 0.50EO   | 0.19 ± 0.06 b  | 23.12 ± 0.48 b,c   | 34.19 ± 0.35 b,c| 307.52 ± 16.09 b                    | 55.95 ± 3.60 a                |
| 0.75EO   | 0.23 ± 0.03 a,b | 23.81 ± 0.40 a,b   | 33.16 ± 1.43 b,c| 279.92 ± 5.73 c                     | 55.66 ± 0.86 a                |
| 1.00EO   | 0.28 ± 0.05 a  | 21.88 ± 0.30 c     | 30.74 ± 1.68 c | 277.2 ± 8.73 c                      | 58.42 ± 4.22 a                |

*abc* Different letters in the same column indicate significant difference by Tukey’s test (*p* ≤ 0.05).

The moisture content, water vapor permeability (WVP) and solubility of the chitosan film reduced significantly (*p* < 0.001) with an increase of EO. The decrease in moisture content, solubility, and WVP has also been reported for films of cassava starch with clove EO [26] and in chitosan films with *Eucalyptus globulus* EO [27]. Jahed et al. [28] observed a significant decrease in the WVP when they incorporated *Carum cuminum* EO in chitosan films. This behavior is attributed to the hydrophobicity of EO in films, which causes low affinity with water molecules [29]. When EO is added to chitosan films, covalent bonds are formed between the functional groups of chitosan and EO chains with a decrease in the availability of hydroxyl and amino groups, limiting the polysaccharide–water interactions for hydrogen bonding [21].

The incorporation of CEO did not significantly affect biodegradability (ranged from 58.42% to 62.65%) of the films over 10 days (Table 2). Similar results were described by Sousa et al. [26], who also observed no effect of adding EO on the biodegradability of cassava starch films with clove EO over 10 days. The present work suggests susceptibility to the rapid action of microorganisms in the biodegradation process.

Optical properties are important in food packaging production because they can affect the first expectation of humans as consumers [30]. Table 3 shows the color parameters of chitosan film with CEO. In the measurement of color, the L* value corresponds to brightness (0—black and 100—white) and the a* and b* parameters correspond to the chromaticity coordinates green (−)/red (+) and blue (−)/yellow (+), respectively [31]. While the a* parameter was not affected (green color with values from −2.41 to −2.46), L* parameter decreased (84.95 to 82.28) and b* parameter increased (24.65 to 30.74) for chitosan films with addition of the CEO.

**Table 3.** Color parameters of chitosan films with *Citrus limonia* essential oil.

| Film     | L*        | a*        | b*        | C*        | h°         |
|----------|-----------|-----------|-----------|-----------|------------|
| Control  | 84.95 ± 0.54 b | −2.46 ± 0.43 a | 24.65 ± 2.49 a | 24.77 ± 1.38 a | 84.30 ± 1.31 a |
| 0.25EO   | 84.68 ± 0.03 b | −2.45 ± 0.72 a | 28.78 ± 0.55 b | 28.88 ± 0.44 b | 85.13 ± 0.73 a |
| 0.50EO   | 83.44 ± 0.06 b | −2.43 ± 0.41 a | 31.19 ± 0.35 b,c | 31.28 ± 0.92 b,c | 85.55 ± 0.71 a |
| 0.75EO   | 83.23 ± 0.03 a,b | −2.43 ± 0.41 a | 33.16 ± 1.43 b,c | 33.25 ± 1.14 c | 85.81 ± 1.40 a |
| 1.00EO   | 82.28 ± 0.05 a | −2.41 ± 0.22 a | 36.74 ± 0.68 d | 36.82 ± 0.34 d | 86.25 ± 0.92 a |

*abc* Different letters in the same column indicate significant difference by Tukey’s test (*p* ≤ 0.05). L*: luminosity, a*: positive means green, negative means reed, b*: positive means yellow, negative means blue, C*: Chroma, h°: hue angle.

The values of the hue angle in this work ranged from 84.30° to 86.25° (between red 0° and yellow 90°), indicating that the films had a yellow color. The chroma values increased significantly (*p* < 0.001) with the CEO concentration from 24.77 to 36.82, indicating that the color of the film became more...
intense. Hafsa et al. [27] added *Eucalyptus globulus* EO in chitosan films and related that the films became darker, red, and yellowish. Song et al. [30] also observed a slight yellowing in the coloring of corn and wheat starch films when they added lemon EO.

The light barrier properties of food films are an important factor in determining the packaging’s ability to protect food from deterioration and loss of nutrients and flavor when it is exposed to visible and ultraviolet light [32]. Figure 1 shows the light barrier properties of the chitosan films with CEO. The chitosan films incorporated with CEO showed good barrier properties to UV light in the region between 200 and 350 nm with a UV light transmission rate below 0%. In the visible region (between 370 and 750 nm), the films with EO proved to be more effective as a barrier to light transmission. These results corroborate those related by Tongnuanchan et al. [33], who also observed a decrease in light transmission, and a consequent increase in opacity, with the addition of EOs in films. This effect can be associated with a dispersion of light by the droplets of essential oil incorporated into the polymeric chitosan matrix [34].

![Figure 1. Light transmission of chitosan films with *Citrus limonia* essential oil.](image)

The quality of foods, such as oils and fats, depends on their oxidative stability [22]. Therefore, it is essential to develop packaging that contributes to their maintenance for delaying the deterioration caused by lipid oxidation, in turn, caused by UV light [34].

The chitosan film is uniform with a flat and crack-free surface (Figure 2). When CEO was added to the chitosan film, especially at higher concentrations, the microstructure was heterogeneous, with oil droplets intimately retained in the continuous network of polysaccharides. Similar behavior has been described for chitosan films with *Eucalyptus globulus* EO [27], films composed of fish gelatin and chitosan with oregano EO [34], and corn starch films incorporated with EO of *Zataria multiflora* Boiss and *Mentha pulegium* [35]. This behavior probably occurred due to the deformation forces that acted during the aggregation of the polymeric chain by evaporation of the solvent [36].

Figure 3 shows the antibacterial activity of the chitosan films with CEO against *Staphylococcus aureus* and *Escherichia coli*. The chitosan films without CEO presented nonantibacterial activity. Although the antimicrobial activity has been reported for chitosan when this material is used to form insoluble films, they cannot be diffused through the agar medium in the agar diffusion test method, and therefore, do not demonstrate antimicrobial properties [21,27,37–39].

Meanwhile, chitosan films with CEO showed significant inhibition (*p* = 0.0247) against *S. aureus*, from the concentration of 0.25% (Figure 3). The zones of inhibition observed were 15.1 and 29.1 mm.
in the chitosan films 0.25EO and 1.0EO, respectively, with significant differences between them ($p = 0.0247$). None of the films studied showed an inhibitory effect against E. coli, a Gram-negative bacterium. Antibacterial activity against S. aureus, the Gram-positive bacteria, may have occurred because these bacteria have a cell envelope structurally and functionally less complex than the envelope of Gram-negative bacteria, as is the case with E. coli. Gram-negative bacteria have lipopolysaccharide molecules on their membranes that act as a barrier, reducing the formation of hydrophobic compounds [40].

![Figure 2](image1.jpg)

**Figure 2.** Micrographics (magnification 1000×) of chitosan films (A) with 0.25% (B), 0.50% (C), 0.75% (D) and 1.0% (E) of *Citrus limonia* essential oil.

![Figure 3](image2.jpg)

**Figure 3.** Antimicrobial activity (mm) of chitosan films with *Citrus limonia* essential oil against *Staphylococcus aureus*. a,b,c,d Different letters in the same column indicate significant difference by Tukey’s test ($p \leq 0.05$).

The antibacterial activity of films with CEO may have occurred because of high limonene content (46.3%) reported in this essential oil (Table 1). The antibacterial potential of limonene was reported in several studies [41–43]. Essential oils contain limonene as the main component. This compound has demonstrated better antibacterial activity against gram-positive strains such as *S. aureus* and *Listeria monocytogenes* [44]. In addition, Araújo et al. [42] evaluated the in vitro antibacterial activity...
of limonene against *P. aeruginosa*, *E. coli*, and *S. aureus*, and they observed that it was more effective against *S. aureus*, as in the present study for the chitosan-film with CEO.

4. Conclusions

A food film and/or coating material composed of chitosan and *Citrus limonia* EO was developed, its characteristics were evaluated, and its antibacterial activity was studied. The results of this study showed that chitosan-based films containing *C. limonia* EO could be used as active films due to their water barrier properties, UV-Vis light and antibacterial activity. The incorporation of *C. limonia* EO in the films decreased the moisture content, solubility, and permeability to water vapor and improved the optical and antibacterial properties, important in food packaging applications. Edible films made with chitosan and *C. limonia* EO provide new ways to improve microbial safety and food shelf life.

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**References**

1. Sagnelli, D.; Hooshmand, K.; Kemmer, G.C.; Kirkensgaard, J.J.K.; Mortensen, K.; Giosafatto, C.V.L.; Holse, M.; Hebelstrup, K.H.; Bao, J.; Stelte, W.; et al. Cross-linked amylose bio-plastic: A transgenic-based compostable plastic alternative. *Int. J. Mol. Sci.* **2017**, 18, 2075. [CrossRef] [PubMed]

2. Giosafatto, C.V.L.; Al-Asmar, A.; D’Angelo, A.; Roviello, V.; Esposito, M.; Mariniello, L. Preparation and characterization of bioplastics from grass pea flour cast in the presence of microbial transglutaminase. *Coatings* **2018**, 8, 435. [CrossRef]

3. Jiménez-Rosado, M.; Zarate-Ramírez, L.S.; Romero, A.; Bengochea, C.; Partal, P.; Guerrero, A. Bioplastics based on wheat gluten processed by extrusion. *J. Clean. Prod.* **2019**, 239, 117994. [CrossRef]

4. Mahcene, Z.; Khelil, A.; Hasni, S.; Akman, P.K.; Bozkurt, F.; Birech, K.; Goudjil, M.B.; Tornuk, F. Development and characterization of sodium alginate based active edible films incorporated with essential oils of some medicinal plants. *Int. J. Biol. Macromol.* **2020**, 145, 124–132. [CrossRef] [PubMed]

5. Di Filippo, M.F.; Panzavolta, S.; Albertini, B.; Bonvicini, F.; Gentilomi, G.A.; Orlacchio, R.; Passerini, N.; Bigi, A.; Dolci, L.S. Functional properties of chitosan films modified by snail mucus extract. *Int. J. Biol. Macromol.* **2020**, 143, 126–135. [CrossRef] [PubMed]

6. de Oliveira Filho, J.G.; Rodrigues, J.M.; Valadares, A.C.F.; de Almeida, A.B.; de Lima, T.M.; Takeuchi, K.P.; Alves, C.C.F.; de Falcão Sousa, H.A.; da Silva, E.R.; Dyszy, F.H.; et al. Active food packaging: Alginate flms with cottonseed protein hydrolysates. *Food Hydrocoll.* **2019**, 92, 267–275. [CrossRef]

7. Sani, I.K.; Pirsa, S.; Tağı, Ş. Preparation of chitosan/zinc oxide/Melissa officinalis essential oil nano-composite film and evaluation of physical, mechanical and antimicrobial properties by response surface method. *Polym. Test.* **2019**, 79, 106004. [CrossRef]

8. Abdelghany, A.M.; Meikhail, M.S.; El-Bana, A.A. Microbial activity and swelling behavior of chitosan/polyvinyl alcohol/sodium alginate semi- natural terpolymer interface containing amoxicillin for wound dressing applications. *Biointerface Res. Appl. Chem.* **2019**, 9, 4368–4373.

9. Priyadarshi, R.; Rhim, J.-W. Chitosan-based biodegradable functional films for food packaging applications. *Innov. Food Sci. Emerg. Technol.* **2020**, 62, 102346. [CrossRef]

10. Ullah, F.; Javed, F.; Khan, A.N.; Kudus, M.H.A.; Jamila, N.; Minhas, A.; Akil, H.M. Synthesis and surface modification of chitosan built nanohydrogel with antiviral and antimicrobial agent for controlled drug delivery. *Biointerface Res. Appl. Chem.* **2019**, 9, 4439–4445.
11. Luis, Â.; Pereira, L.; Domingues, F.; Ramos, A. Development of a carboxymethyl xylan film containing licorice essential oil with antioxidant properties to inhibit the growth of foodborne pathogens. *LWT Food Sci. Technol.* 2019, 111, 218–225. [CrossRef]

12. Mir, S.A.; Dar, B.N.; Wani, A.A.; Shah, M.A. Effect of plant extracts on the techno-functional properties of biodegradable packaging films. *Trends Food Sci. Technol.* 2018, 80, 141–154. [CrossRef]

13. Yeddes, W.; Djebali, K.; Aïdi Wannes, W.; Hammami, M.; Younes, L.; Saidani Tounsi, M. Gelatin-chitosan-pectin films incorporated with rosemary essential oil: Optimized formulation using mixture design and response surface methodology. *Int. J. Biol. Macromol.* 2020, 154, 92–103. [CrossRef] [PubMed]

14. Amalraj, A.; Haponiuk, J.T.; Thomas, S.; Gopi, S. Preparation, characterization and antimicrobial activity of polyvinyl alcohol/gum arabic/chitosan composite films incorporated with black pepper essential oil and ginger essential oil. *Int. J. Biol. Macromol.* 2020, 151, 366–375. [CrossRef] [PubMed]

15. Salvia-Trujillo, L.; Rojas-Graü, A.; Soliva-Fortuny, R.; Martín-Belloso, O. Physicochemical characterization and antimicrobial activity of food-grade emulsions and nanoemulsions incorporating essential oils. *Food Hydrocoll.* 2015, 43, 547–556. [CrossRef]

16. Tariq, S.; Wani, S.; Rasool, W.; Shafi, K.; Bhat, M.A.; Prabhakar, A.; Shalla, A.H.; Rather, M.A. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. *Microb. Pathog.* 2019, 134, 103580. [CrossRef] [PubMed]

17. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods–A review. *Int. J. Food Microbiol.* 2004, 94, 223–253. [CrossRef]

18. Nerio, L.S.; Olivero-Verbel, J.; Stashenko, E. Repellent activity of essential oils: A review. *Bioresour. Technol.* 2010, 101, 372–378. [CrossRef]

19. Pires, T.C.; Piccoli, R.H. Inhibitory effect of essential oils from the genus Citrus on the microorganisms growth. *Rev. Inst. Adolfo Lutz* 2012, 71, 378–385.

20. Estevam, E.B.B.; Miranda, M.L.D.; Alves, J.M.; Egea, M.B.; Pereira, P.S.; Martins, C.H.G.; Esperandim, V.R.; Magalhães, L.G.; Bolela, A.C.; Cazal, C.M.; et al. Chemical composition and biological activities of the essential oils from the fresh leaves of Citrus limonia osbeck and Citrus latifolia Tanaka (Rutaceae). *Rev. Virtual Quim.* 2016, 8, 1842–1854. [CrossRef]

21. Ojagh, S.M.; Rezaei, M.; Razavi, S.H.; Hosseini, S.M.H. Development and evaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water. *Food Chem.* 2010, 122, 161–166. [CrossRef]

22. Stoll, L.; da Silva, A.M.; Silva Lahnke, A.O.E.; Costa, T.M.H.; Flóres, S.H.; de Oliveira Rios, A. Active biodegradable film with encapsulated anthocyanins: Effect on the quality attributes of extra-virgin olive oil during storage. *J. Food Process. Preserv.* 2017, 41, 1–8. [CrossRef]

23. Casariego, A.; Souza, B.W.S.; Cerqueira, M.A.; Teixeira, J.A.; Cruz, L.; Diaz, R.; Vicente, A.A. Chitosan/clay films’ properties as affected by biopolymer and clay micro/nanoparticles’ concentrations. *Food Hydrocoll.* 2009, 23, 1895–1902. [CrossRef]

24. Kavoosi, G.; Rahmatollahi, A.; Mohammad Mahdi Dadfar, S.; Mohammadi Purfard, A. Effects of essential oil on the water binding capacity, physico-mechanical properties, antioxidant and antibacterial activity of gelatin films. *LWT Food Sci. Technol.* 2014, 57, 556–561. [CrossRef]

25. Nisar, T.; Wang, Z.C.; Yang, X.; Tian, Y.; Iqbal, M.; Guo, Y. Characterization of citrus pectin films integrated with clove bud essential oil: Physical, thermal, barrier, antioxidant and antibacterial properties. *Int. J. Biol. Macromol.* 2018, 106, 670–680. [CrossRef]

26. de Figueiredo Sousa, H.A.; Oliveira Filho, J.G.; Egea, M.B.; da Silva, E.R.; Macagnan, D.; Pires, M.; Peixoto, J. Active film incorporated with clove essential oil on storage of banana varieties. *Nutr. Food Sci.* 2019, 49, 911–924. [CrossRef]

27. Hafsa, J.; ali Smach, M.; Ben Khedher, M.R.; Charfeddine, B.; Limem, K.; Majdoub, H.; Rouatbi, S. Physical, antioxidant and antimicrobial properties of chitosan films containing Eucalyptus globulus essential oil. *LWT Food Sci. Technol.* 2016, 68, 356–364. [CrossRef]

28. Jahed, E.; Khaledabad, M.A.; Almasi, H.; Hasanzadeh, R. Physicochemical properties of Carum copticum essential oil loaded chitosan films containing organic nanoreinforcements. *Carbohydr. Polym.* 2017, 164, 325–338. [CrossRef]
29. Ma, Q.; Zhang, Y.; Critzer, F.; Davidson, P.M.; Zivanovic, S.; Zhong, Q. Physical, mechanical, and antimicrobial properties of chitosan films with microemulsions of cinnamon bark oil and soybean oil. Food Hydrocoll. 2015, 52, 533–542. [CrossRef]

30. Song, X.; Zuo, G.; Chen, F. Effect of essential oil and surfactant on the physical and antimicrobial properties of corn and wheat starch films. Int. J. Biol. Macromol. 2018, 107, 1302–1309. [CrossRef]

31. Adilah, Z.A.M.; Jamilah, B.; Hanani, Z.A.N. Functional and antioxidant properties of protein-based films incorporated with mango kernel extract for active packaging. Food Hydrocoll. 2018, 74, 207–218. [CrossRef]

32. De Moraes Crizel, T.; de Oliveira Rios, A.; Alves, V.D.; Bandarra, N.; Moldão-Martins, M.; Flôres, S.H. Active food packaging prepared with chitosan and olive pomace. Food Hydrocoll. 2018, 74, 139–150. [CrossRef]

33. Tongnuanchan, P.; Benjakul, S.; Prodpran, T. Properties and antioxidant activity of fish skin gelatin film incorporated with citrus essential oils. Food Chem. 2012, 134, 1571–1579. [CrossRef] [PubMed]

34. Hosseini, S.F.; Rezaei, M.; Zandi, M.; Farahmandghavi, F. Bio-based composite edible films containing Origanum vulgare L. essential oil. Ind. Crops Prod. 2015, 67, 403–413. [CrossRef]

35. Ghasemlou, M.; Aliheidari, N.; Fahmi, R.; Shojaee-Aliabadi, S.; Keshavarz, B.; Cran, M.J.; Khaksar, R. Physical, mechanical and barrier properties of corn starch films incorporated with plant essential oils. Carbohydr. Polym. 2013, 98, 1117–1126. [CrossRef] [PubMed]

36. Sánchez-González, L.; Vargas, M.; González-Martínez, C.; Chiralt, A.; Cháfer, M. Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil. Food Hydrocoll. 2009, 23, 2102–2109. [CrossRef]

37. Ouattara, B.; Simard, R.E.; Piette, G.; Bégin, A.; Holley, R.A. Diffusion of acetic and propionic acids from chitosan-based antimicrobial packaging films. J. Food Sci. 2000, 65, 768–773. [CrossRef]

38. Zivanovic, S.; Chi, S.; Draughon, A.E. Antimicrobial Activity of Chitosan films enriched with essential oils. J. Food Sci. 2005, 70, 45–51. [CrossRef]

39. Wang, L.; Liu, F.; Jiang, Y.; Chai, Z.; Li, P.; Cheng, Y.; Jing, H.; Leng, X. Synergistic antimicrobial activities of natural essential oils with chitosan films. J. Agric. Food Chem. 2011, 59, 12411–12419. [CrossRef]

40. Otoni, C.G.; da Moura, M.R.; Aouada, F.A.; Camilloto, G.P.; Cruz, R.S.; Lorevice, M.V.; de FF Soares, N.; Mattoso, L.H.C. Antimicrobial and physical-mechanical properties of pectin/papaya puree/cinnamaldehyde nanoemulsion edible composite films. Food Hydrocoll. 2014, 41, 188–194. [CrossRef]

41. Lu, H.; Xu, C.; Zhang, X.; Liang, Y.; Liu, X. Antibacterial effect of limonene on food-borne pathogens. J. Agric. Life Sci. 2016, 42, 306–312.

42. de Araújo, A.C.J.; Freitas, P.R.; dos Santos Barbosa, C.R.; Muniz, D.F.; Rocha, J.E.; da Silva, A.C.A.; de Morais Oliveira-Tintino, C.D.; Ribeiro-Filho, J.; da Silva, L.E.; Confortin, C.; et al. GC–MS–FID characterization and antibacterial activity of the Mikania cordifolia essential oil and limonene against MDR strains. Food Chem. Toxicol. 2020, 136, 111023. [CrossRef] [PubMed]

43. Espina, L.; Gelaw, T.K.; de Lamo-Castellvi, S.; Pagán, R.; García-Gonzalo, D. Mechanism of Bacterial Inactivation by (+)-Limonene and Its Potential Use in Food Preservation Combined Processes. PloS ONE 2013, 8, 1–10. [CrossRef] [PubMed]

44. Settanni, L.; Palazzolo, E.; Guarrasi, V.; Aleo, A.; Mammina, C.; Moschetti, G.; Germanà, M.A. Inhibition of foodborne pathogen bacteria by essential oils extracted from citrus fruits cultivated in Sicily. Food Control 2012, 26, 326–330. [CrossRef]