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Posted Date: 9 June 2023

doi: 10.20944/preprints202306.0681.v1

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Thymol@Natural Zeolite Nanohybrids for Chitosan/Poly-vinyl-alcohol Based Hydrogels Applied as Active Pads for Strawberries Preservation

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Abstract: Food saving and further valorization is of major interest in our days. Worldwide, scientists intensify their efforts to increase shelf life of products due to the global danger of coming food crisis. Even though many chemicals could achieve such target, the circular economy spirit and the global trend against the greenhouse effects impose the turn of research activities using environmentally friendly biomass materials, biowastes, and biodegradable materials. In this study, the promising biopolymer chitosan incorporated with the promising biodegradable polymer poly-vinyl-alcohol to produce a biopolymeric matrix. This biodegradable biopolymer was further mixed homogeneously with 15% thymol/nano-zeolite nanohybrid material and the final developed film was extremely improved compared to the relevant of chitosan/poly-vinyl-alcohol film. Mechanical properties were increased significantly i.e., 34% for Young Modulus and 46,5% for ultimate tensile strength, while the antioxidant activity increased by 53,4%. The antibacterial activity increased by 134% for *Escherichia coli*, 87,5% for *Staphylococcus aureus*, 32% for *Listeria monocytogenes*, and 9% for *Salmonella enterica*. The water-vapor diffusion coefficient and the oxygen permeability coefficient decreased down to -51% and -74% respectively, and thus the water-vapor and oxygen barrier increased. In-vivo experiments on strawberries show an extension of 233% for shelf-life duration which resulted from the modified atmosphere by the control-released thymol.

Keywords: chitosan; poly-vinyl-alcohol; natural zeolite; thymol; active packaging; antibacterial activity; strawberry preservation

1. Introduction

Nowadays, fruit and vegetables industries produce a large amount of waste due to overproduction and short shelf-life of such products [1]. Following the circular economy spirit, different strategies were proposed to reduce such waste. One of the dominant proposed practices is the increase of fruit and vegetables shelf-life, and thus the extend of time that consumers can buy and consume fruit and vegetables [2]. This method could also help to keep nutrition value of vegetables and fruits high [3,4]. The use of polysaccharide based edible films and coatings as novel nontoxic, renewable, and degradable materials to preserve fruits and control their aerobic respiration, is also a proposed method, which is follows the sustainability and circular economy model [5,6]. Recent studies shown that polysaccharide-based films and coating control and retard fruits; and vegetables respiration rates. Furthermore, they provide the amount of water which is necessary for the preservation of such foods [7].

Cellulose, casein, zein, soy protein, and chitosan are some of the several possible edible coatings for fruits. A polysaccharide-based film must be odorless, tasteless, and transparent in order to be used as a fruit-based coating [8]. For such edible coatings it is not easy to measure the gas permeation after being placed on fruits. For this purpose, usually, separate flat films need to be prepared and tested. Chitosan (CS) has a great potential to be used as alternative film and coating in the future as it is derived from the second most abundant biopolymer, the chitin [9,10]. CS is biodegradable, non-toxic, and it has significant antibacterial properties against gram positive and gram-negative bacteria [10]. CS has also excellent film forming properties, good mechanical properties, and selective permeability to gases such as CO_2 and O_2 [11,12]. Even though CS is general recognized as safe (GRAS) it is still not allowed to be used as a food additive [13].

Poly-vinyl-alcohol (PVOH) is a water soluble, biodegradable synthetic biopolymer which has been shown to have a great mixability and compatibility with CS to develop CS/PVOH food packaging films [14]. In advance it has been previously shown that PVOH could enhance the antibacterial activity of CS against various food pathogens [15,16].

Essential oils (EO) are provided as alternative naturally abundant antioxidant and antibacterial components that could be used as sustainable food additives and food preservatives in the food packaging sector [17,18].

In the literature, there are a plethora of studies providing CS/EO-based films for food packaging films and coating applications [19]. A new technology towards the use of EO as novel food preservatives in polymer and biopolymer films and coatings provides control of their release by immobilizing them on naturally abundant adsorbents such as nanoclays [19–22]. Recently zeolites have been proposed as an alternative nanoreinforcement raw material for food packaging applications [23]. Natural zeolites (NZ) have been also proposed as a potential nanocarrier for EO control release in pork fillets food packaging films [24] and kiwi fruits coatings [16].

Although strawberry (*Fragaria ananassa*), is a fruit with high nutritional value it has a short shelf-life due to water loss, texture softening, physiological deterioration and microbiological decay [25–27]. In the literature, there are many studies with EO incorporated into CS-based films, which have been applied as coatings and succeeded in preserving and maintaining the quality of strawberries [28–32]. Based on the available literature, no reference has been found reporting a process where thymol (TO) is initially absorbed into NZ to create a TO@NZ nanohybrid, and this nanohybrid, once formed, to be dispersed within a CS/PVOH matrix. This procedure produced active pads which released TO in a controlled manner in the packaging environment, and this package was tested for the preservation of strawberries.

In this study novel CS/PVOH based films with NZ and NZ modified with thymol (TO@NZ) are developed via a solution casting method. The content of NZ and TO@NZ on CS/PVOH based films varied from 5, 10 and 15 wt%. The obtained CS/PVOH/xNZ and CS/PVOH/xTO@NZ films (x = 5, 10 and 15) were physiochemically characterized with XRD analysis, and FTIR spectroscopy. In advance, the tensile, water/oxygen barrier, antioxidant, and antibacterial properties of such CS/PVOH/xNZ and CS/PVOH/xTO@NZ films were studied. The most active CS/PVOH/xNZ and CS/PVOH/xTO@NZ films were applied as strawberries active pads. Here are the points of innovation of the current work: (i) CS/PVOH/xNZ and CS/PVOH/xTO@NZ active films first time developed and characterized, (ii) such CS/PVOH/xNZ and CS/PVOH/xTO@NZ films first time applied as strawberries active pads.

2. Results

2.1. XRD analysis of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

In Figure 1 the XRD plots of all obtained CS/PVOH/xNZ and CS/PVOH/xTO@NZ films as well as pure CS/PVOH film are shown for comparison.

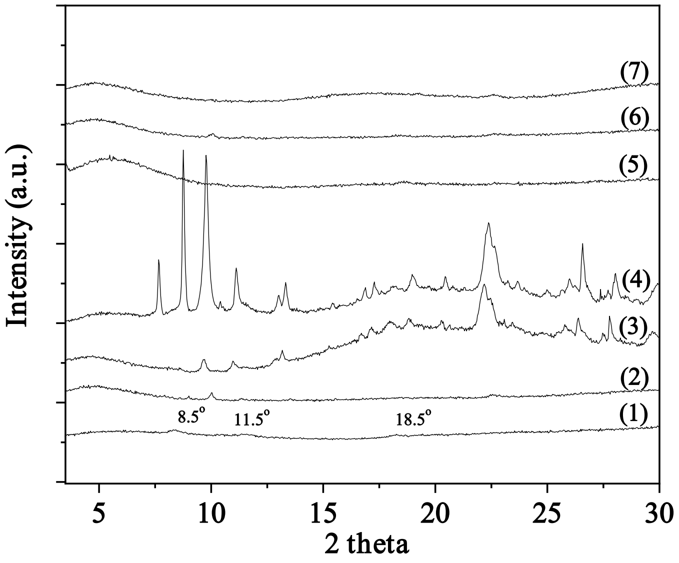


Figure 1. XRD plots of (1) CS/PVOH, (2) CS/PVOH/5NZ (3) CS/PVOH/10NZ, (4) CS/PVOH /15NZ, (5) CS/PVOH /5TO@NZ, (6) CS/PVOH/10TO@NZ, and (7) CS/PVOH /15TO@NZ obtained films.

As it is observed in Figure 1 (see plot line (1)) the XRD plot of pure CS/PVOH corresponds to an almost amorphous biopolymer structure. Only the broad reflections of CS at around 8.5°, 11.5° and 18.5° 2 theta are observed which correspond hydrated crystal structure of CS [15,16]. In the case of CS/PVOH/xNZ films plots (see plot lines (2), (3) and (4)) the characteristic peaks of NZ are observed which are attributed to the Heulandite $Ca(Si_7Al_2)O_{16}\ 6H_2O$ monoclinic crystal phase (PDF-41-1357). As the wt.% content of NZ is increased the characteristic peaks of NZ are increased farther. This suggests a non-uniform dispersion of NZ in CS/PVOH matrix and that aggregates of NZ are probably obtained. In the case of CS/PVOH/xTO@NZ films plots (see plot lines (5), (6) and (7)) the characteristic peaks of NZ were not observed suggesting a homogeneous dispersion of modified TO@NZ in CS/PVOH matrix supported by the TO molecules adsorbed in the NZ.

2.2. FTIR spectroscopy of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

In Figure 2 the FTIR spectra of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films as well as pure CS/PVOH film are observed for comparison.

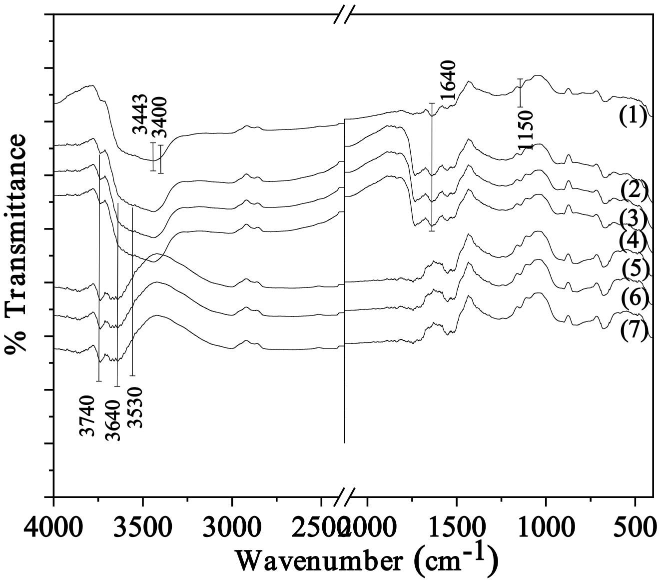


Figure 2. FTIR plots of (1) CS/PVOH, (2) CS/PVOH/5NZ (3) CS/PVOH/10NZ, (4) CS/PVOH /15NZ, (5) CS/PVOH /5TO@NZ, (6) CS/PVOH/10TO@NZ, and (7) CS/PVOH /15TO@NZ obtained films.

FTIR spectra of pure CS/PVOH film (see line (1) in Figure 2) is a combination of both CS and PVOH reflections. The large band at 3443 cm^{-1} is assigned to stretching vibration of hydroxyl groups of both CS and PVOH. The band at 3400 cm^{-1} is assigned the primary stretching vibration of amino groups of CS. The same band is also assigned to the intra- and inter-molecular hydrogen bonds of the CS/PVOH matrix [16,33]. The band at 1637–1644 cm^{-1} is assigned to the associated water, C–OH from the glycosidic units of CS chains and to the vibration of carboxamide O= C–NHR of CS [16,34]. The band at 1150 cm^{-1}, is assigned to the asymmetric bridge stretch of the glycosidic linkage of CS [16].

In the FTIR spectra of CS/PVOH/xNZ films (see lines (2), (3), (4) in Figure 2) the stretching reflections of NZ’s hydroxyl groups at 3740 cm^{-1}, 3640 and 3540 cm^{-1} are also observed additionally to the presence of CS/PVOH reflections. According to Tvaruskova and Bosacek [35] the band at 3740 cm^{-1} is independent of the degree of cationization of NZ. This band is attributed to the terminal hydroxyl group in the NZ crystal. The band at 3640 cm- 1, denoted as a high-frequency (HF) band, is narrow and symmetrical and its intensity depends on the degree of decationization. It was found that these hydroxyl groups are located in large cavities of the Y zeolite; hence they are easily accessible and can be affected by sorption of saturated and unsaturated hydrocarbons [36]. The band at 3550 cm^{-1}, denoted as a low-frequency (LF) band, is broad, asymmetrical and it also depends on the degree of decationization. The hydroxyl groups corresponding to this band are located in sodalite units of the zeolite structure and, although we assume that the protons in these groups are more loosely bound than those in the previous case, these hydroxyl groups are not sensitive with respect to the sorption of nonpolar molecules or olefins because of their inaccessibility [35].

In the FTIR spectra of CS/PVOH/xTO@NZ films (see lines (5), (6), (7) in Figure 2) three are the main differences in comparison to the FTIR spectra of CS/PVOH/xNZ films: (i) the increase of the band at 3640 cm^{-1} of NZ hydroxyl groups, (ii) the attenuation of amino and hydroxyl group bands of CS/PVOH matrix at 3400-3443 cm^{-1} and (iii) the attenuation of the band at 1640 cm^{-1} of CS/PVOH matrix. The increase of the hydroxyl group band at 3640 cm^{-1} could be attributed to the adsorbed TO molecules on these sites according to the information mentioned hereabove about the ability of these hydroxyl group sites to adsorb saturated or unsaturated hydrocarbons. The attenuation of the bans at 3400-3443 cm^{-1} and 1640 cm^{-1} of CS/PVOH matrix could be attributed to the interplay between CS/PVOH matrix and modified TO@NZ nanohybrid.

In any case the FTIR spectra suggests the higher interplay and relaxation between CS/PVOH matrix and modified TO@NZ nanohybrid than CS/PVOH matrix and pure NZ. This result is in accordance with XRD results shown hereabove and suggested higher dispersion of TO@NZ nanohybrid into CS/PVOH matrix than pure NZ.

2.3. Tensile properties of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

In Figure 3 the representative stress-strain curves of all CS/PVOH/xNZ and CS/PVOH/xTO@NZ films as well as pure CS/PVOH film are shown for comparison. From these stress strain curves the Young's (E) modulus, ultimate tensile strength (σ_{uts}), and % strain at break (εb) have been calculated and are listed in Table 1 for comparison.

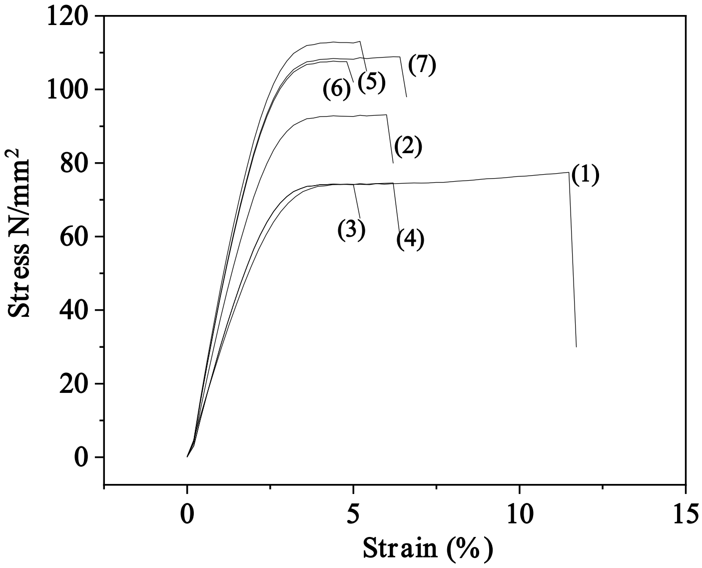


Figure 3. Stress-strain curves of (1) CS/PVOH, (2) CS/PVOH/5NZ (3) CS/PVOH/10NZ, (4) CS/PVOH /15NZ, (5) CS/PVOH /5TO@NZ, (6) CS/PVOH/10TO@NZ, and (7) CS/PVOH /15TO@NZ obtained films.

Table 1. Calculated values of Young's (E) modulus, ultimate tensile strength (σ_{uts}), and % strain at break (ε_b).

	E	σ uts	ε%
CS/PVOH	2249.3(200.3)	71.2(1.8)	11.8(0.9)
CS/PVOH/5NZ	3064.3(26.3)	89.0(4.6)	6.9(1.1)
CS/PVOH/10NZ	2736.0(351.3)	71.0(16.7)	6.7(2.8)
CS/PVOH/15NZ	2803.4(345.3)	73.3(4.5)	7.0(2.1)
CS/PVOH/5TO@NZ	3304.0(279.5)	109.3(17.2)	6.8(1.2)
CS/PVOH/10TO@NZ	3186.5(125.2)	103.7(1.4)	6.7(2.3)
CS/PVOH/15TO@NZ	3010.0(481.3)	104.7(5.5)	7.1(2.2)

From the values of the listed in Table 1 Young's (E) modulus, ultimate tensile strength (σ_{uts}), and % strain at break (ε_b) values it is revealed that the addition of pure NZ in CS/PVOH matrix increases Young's Modulus values and decreases elongation at break values. At the same time, the ultimate strength increases only in the case of CS/PVOH/5NZ film and remains unchanged for CS/PVOH/10NZ and CS/PVOH/15NZ films. This behavior is typical for rigid inorganic materials such as NZ blended with a polymer [37,38].

On the contrary, when modified TO@NZ nanohybrid added in CS/PVOH matrix both stress and ultimate strength values increased while elongation at break values decreased. This behavior indicates that during the modification with TO@NZ nanohybrids, the TO molecule acts as a kind of a compatibilizer and succeeds higher incorporation in CS/PVOH matrix than the pure NZ. The results of tensile properties, combined with XRD analysis and FTIR spectrometry, show a higher dispersion and a higher relaxation of TO@NZ nanohybrid with CS/PVOH matrix as compared to the relevant properties of pure NZ respectively.

2.4. Water/oxygen barrier properties of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

In Table 2 the obtained water-oxygen transmission rate values (WVTR) and the oxygen transmission rate (OTR) values for all CS/PVOH/xNZ and CS/PVOH/xTO@NZ films as well as pure CS/PVOH film are listed. From WVTR and OTR values along with film thickness, the water diffusivity (D_w) and the oxygen permeability (PeO_2) values are calculated and are listed in Table 2 also for comparison.

Table 2. Film thickness, water vapor transmission rate (WVTR), water diffusivity (D_W), oxygen transmission rate (OTR), and oxygen permeability (PeO_2) values of pure CS/PVOH film as well as of CS/PVOH/HNT and CS/PVOH/TO@HNT films.

	Film thickness (mm)	Water Vapor Transmition Rate (10^{-6} g/cm^2.day)	D_w - Water Diffusion Coefficient (10^{-4} cm^2/s)	Oxygen Transmition Rate (ml/m^2.day)	Pe_{O2} (10^{-7}cm^2/s)
CS/PVOH	0.17	1.06(0.12)	3.65(0.11)	38.2(0.2)	6.5(0.3)
CS/PVOH/5NZ	0.11	1.17(0.10)	3.21(0.09)	33.7(0.2)	3.7(0.1)
CS/PVOH/10NZ	0.12	1.01(0.13)	2.67(0.12)	26.1(0.2)	2.6(0.1)
CS/PVOH/15NZ	0.10	0.92(0.09)	2.33(0.08)	27.5(0.3)	2.2(0.2)
CS/PVOH/5TO@NZ	0.11	0.92(0.07)	2.03(0.06)	15.5(0.2)	1.8(0.2)
CS/PVOH/10TO@NZ	0.08	0.81(0.07)	1.6(0.05)	34.4(0.2)	3.4(0.1)
CS/PVOH/15TO@NZ	0.10	0.79(0.05)	1.8(0.04)	15.0(0.2)	1.7(0.2)

By both D_w and PeO_2 values it is obvious that the addition of both pure NZ and modified TO@NZ nanohybrid reduces water and oxygen permeability. As the wt.% content of NZ and TO@NZ increase the water and oxygen permeability increase farther. Higher increase of water/oxygen barrier is obtained for TO@NZ based samples than pure NZ based samples. Thus, both pure NZ and modified TO@NZ are good barrier nanofillers while the modified TO@NZ nanohybrid prevails to pure NZ.

2.5. Total antioxidant activity of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

Antioxidant activity for such active packaging films is a crucial parameter to extend the shelf - life of foods and preserve their nutritional and their aesthetic quality by delaying the deterioration of food whichtakes place through oxidation reactions.

The calculated % total antioxidant activity values of all CS/PVOH/xNZ and CS/PVOH/xTO@NZ films as well as pure CS/PVOH film are plotted in Figure 4.

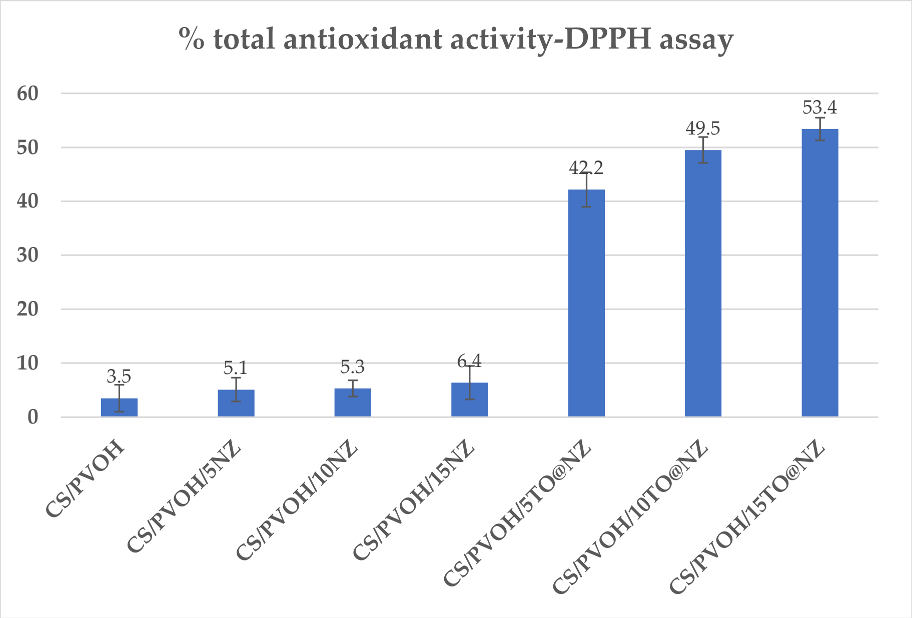


Figure 4. % Total antioxidant activity of all obtained CS/PVOH/xNZ and CS/PVOH/xTO@NZ films.

As it was expected no significant antioxidant activity is obtained for pure CS/PVOH and CS/PVOH/xNZ films. For CS/PVOH/xTO@NZ films antioxidant activity is increased as the TO@NZ wt.% content is increased. The highest antioxidant activity value is equal to 53.4% and is obtained for CS/PVOH/15TO@NZ film.

2.6. Antibacterial properties of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

The antibacterial efficacy of the investigated nanoreinforcement CS/ PVOH based packaging films is presented in Table 3 and Figure 5.

The antibacterial activity of the different film materials was assessed against four foodborne pathogenic bacteria: *Escherichia Coli* (*E. coli*), *Staphylococcus aureus (S. aureus), Salmonella. Enterica (S. Enterica) and Listeria monocytogenes (L. monocytogenes)*. The inhibitory activity of the film materials was assessed by measuring the diameter of the clear inhibition zone formed around the agar wells. In cases where no clear zone was observed surrounding the agar wells, it was interpreted as the absence of an inhibitory zone, and the diameter was recorded as zero.

Table 3. Antimicrobial activity of active films against food pathogenic bacteria *E. coli*, *S. aureus*, *S. enterica* and *L. monocytogenes*.

Film material	*E. coli*	*S. aureus*	*S. enterica*	*L. monocytogenes*
	Inhibition[1] (diameter of clear zone)	Inhibition[1] (diameter of clear zone)	Inhibition[1] (diameter of clear zone)	Inhibition[1] (diameter of clear zone)
CSPVOH	3.57 ± 0.55	4.23 ± 0.48	3.26 ± 0.17	3.40 ± 0.70
CSPVOH5NZ	0.00	0.00	0.00	0.00
CSPVOH10NZ	0.00	0.00	0.00	0.00
CSPVOH15NZ	0.00	0.00	0.00	0.00
CSPVOH5%TO@NZ	3.93 ± 0.53	4.73 ± 0.15	3.37 ± 0.16	3.70 ± 0.14
CSPVOH10TO@NZ	5.35 ± 0.30	5.32 ± 0.19	3.53 ± 0.18	4.05 ± 0.18
CSPVOH15TO@NZ	8.35 ± 0.45	7.93 ± 0.54	3.55 ± 0.07	4.48 ± 0.08

[1] Inhibitory zone surrounding film discs measured in mm after the subtraction of the well diameter (6mm). Result Results expressed as mean ± standard deviation (n=3). Means in the same column baring different superscript letters are significantly different ($p<0.5$).

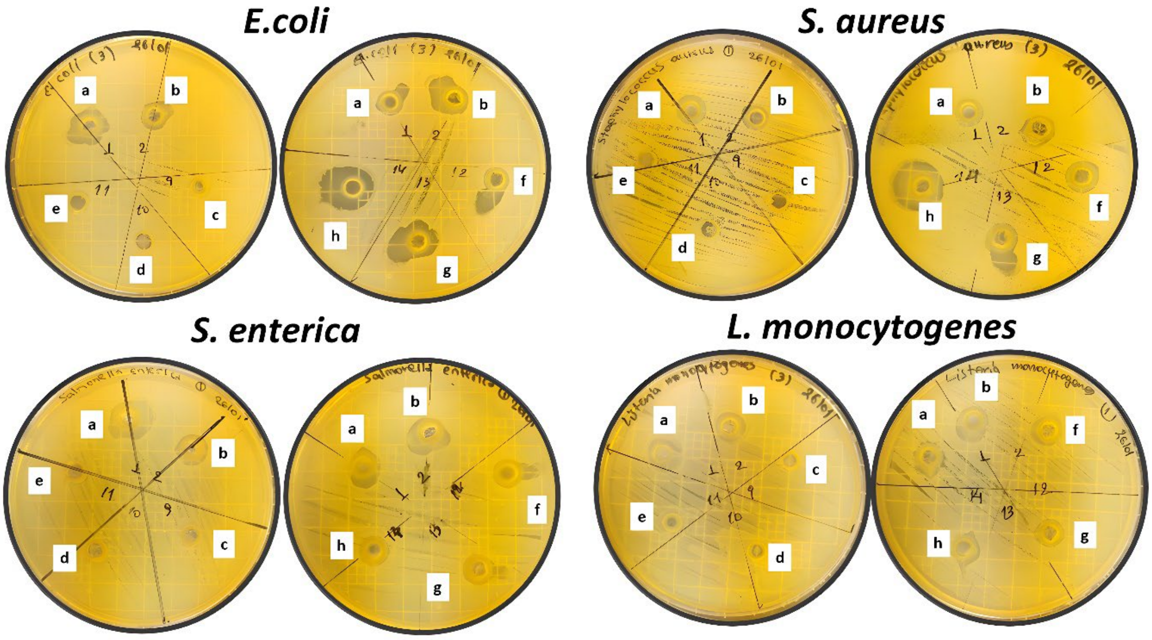


Figure 5. Petri dishes images of (a) CS, (b) CS/PVOH, (c) CS20PVOH 5%NZ, (d) CS20PVOH 10%NZ, (e) CS20PVOH 15%NZ, (f) CS20PVOH 5%TO@NZ, (g) CS20PVOH 10%TO@NZ, (h) CS20PVOH 15%TO@NZ films against *E. coli*, *S. aureus*, *S. enterica*, and *L. monocytogenes*.

The film material CS/PVOH displayed moderate antibacterial activity. Notably, the inhibition zone diameters against *E. coli and S. aureus* were 3.57 ± 0.55 and 4.23 ± 0.48 respectively, suggesting efficacy against these bacterial strains. The antimicrobial activity against *S. enterica* and *L. monocytogenes* were 3.26 ± 0.16 and 3.40 ± 0.70 respectively. This outcome was expected since it is widely recognized that CS exhibits antibacterial activity, which can be attributed to the interaction between the positively charged ammonium (NH_4^+) groups present in the amino glucose units of CS and the negatively charged components of the bacterial cell wall. This interaction contributes to the antibacterial effects displayed by CS against various microorganisms [39]. In advance as it was shown recently antibacterial activity of CS was supported and enhanced by PVOH presence [16,40].

Conversely, the film materials incorporating zeolite (CS/PVOH/NZ, CS/PVOH/10NZ, and CS/PVOH/15NZ) did not demonstrate observable inhibition zones against any of the tested bacteria. Furthermore, these three film materials were also subjected to testing their antimicrobial activity using the disc diffusion method to investigate if they exhibited any activity when brought into direct contact with the inoculated agar surface. The findings revealed that despite the absence of an inhibition zone, there was evident antimicrobial activity observed at the contact area. The absence of the diffusion activity indicates that the antimicrobial compounds present in the films did not effectively spread any more throughout the surrounding medium when zeolite added. Therefore, the inhibitory effect was primarily localized to the immediate vicinity of the films. Further research is warranted to investigate the factors influencing the diffusion capability of the antimicrobial compounds and to optimize the film formulation for enhanced antimicrobial activity.

On the other hand, all the tested materials containing thyme oil encapsulated with zeolite (CS/PVOH/5TO@NZ, CS/PVOH/10TO@NZ, and CS/PVOH/15TO@NZ) exhibited notable antimicrobial activity. Against *E. coli*, the resulting inhibition zone diameters were 3.93 ± 0.53, 5.35 ± 0.30, and 8.35 ± 0.45 respectively. Similarly, for S. aureus, the inhibition zone diameters were 4.73 ± 0.15, 5.32 ± 0.19, and 7.93 ± 0.54. These findings suggest that the inclusion of thyme oil encapsulated with zeolite enhanced the antimicrobial effectiveness of the film materials against to the four mentioned kinds of microbes, and particularly against *S. aureus and E. coli*.

In summary, the film materials incorporating thymol encapsulated with zeolite, demonstrated the highest antimicrobial activity. Conversely, the film materials containing zeolite alone did not exhibit significant antimicrobial efficacy. This finding aligns with existing literature, which reports the lack of inherent antibacterial activity exhibited by zeolite itself [41,42].

Zeolite is characterized by its distinctive framework structure, consisting of interconnected channels and cavities. Within this framework, exchangeable cations help maintain a balance by compensating for the permanent negative charge resulting from isomorphous substitution. The porous nature of zeolites enables them to adsorb water molecules, providing hydration to the exchangeable cations located within the framework. One of the remarkable properties of zeolites is their exceptional sorption capacity. They can accumulate various compounds, including water and salts. This sorption capability allows zeolites to serve as carriers for active substances, such as antibacterial and antifungal compounds. These active compounds can be incorporated into the zeolite structure, taking advantage of the porous framework for controlled release over time [43].

In the present study, the variation in antimicrobial activity observed among the tested bacteria and the different films can be attributed mostly to the amount of loaded bioactive compound (thyme oil) onto film materials. It is well-documented that higher loadings of essential oils results in stronger antimicrobial activity.

Comparable results were observed, in another study, where thymol-impregnated starch-chitosan-zeolite films, with thymol concentrations of 24% and 27%, exhibited significant antibacterial effects against *S. aureus and E. coli*. This finding indicated that thymol was successfully released from the film into the surrounding culture medium, while maintaining its antibacterial activity after impregnation within the polymer matrix [44].

Similarly, Pajnik et al. (2022) [42] reported analogous findings regarding the antibacterial effects of Zeolite/Chitosan/Gelatin films. They observed that the antibacterial effect of the films alone, without thymol or carvacrol impregnation, was insignificant. However, when thymol or carvacrol was incorporated into the films, strong antibacterial activity was observed against both bacterial strains [42].

The antibacterial mechanism of phenolic compounds like TO is associated with their ability to disrupt the cell wall and membranes of bacteria. This disruption can lead to cell lysis and the release of cellular contents. Thymol, in particular, is known to integrate with the polar head-groups of the lipid bilayer, inducing alterations in the cell wall [45].

Moreover, the antimicrobial activity of zeolites can be influenced by various factors such as the type, size, structure, physical appearance, the nature of the incorporated compound and loading concentration of the zeolite. Additionally, the specific microorganisms targeted, and the environmental conditions can also affect the effectiveness of zeolites as antimicrobial agents. Therefore, it is important to consider these factors when utilizing zeolites for antimicrobial applications [46].

The combination of zeolites' unique framework structure, exchangeable cations, and porous nature allows for effective adsorption and release of active substances. This, in conjunction with the antimicrobial properties of active substances, allows zeolites to exert their antimicrobial effects. Encapsulation of essential oils (EO) in zeolites offers advantages in terms of physical stability, reduced volatility, and protection against light, humidity, and pH variations. This encapsulation also enables controlled release of the EO under suitable conditions. Zeolites' properties, such as their crystal size, morphology, porosity, and chemical composition, contribute to the successful encapsulation of EO. Furthermore, zeolites exhibit good biocompatibility, low toxicity, and enhanced access to the micropores, making them suitable carriers for EO compounds. However, due to the high volatility, low photostability, and thermolability of EOs, encapsulation in zeolites often may requires the use of high-concentration solutions in order to exhibit a significant activity [47].

Overall, zeolites have demonstrated promising antimicrobial activity and hold potential for various applications in healthcare, environmental, and food safety sectors. Further research and development are ongoing to explore and optimize the antimicrobial properties of zeolites and their practical applications. These findings contribute valuable insights toward the development of antimicrobial film materials for potential applications in food packaging and related industries.

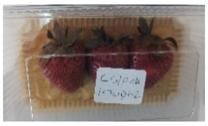
2.7. Preservation of fresh Strawberries-protection against mold of fungi

Representative photos of packed strawberries inside the PP boxes uncoated (blank sample) and coated with CS/PVOH, CS/PVOH/15NZ and CS/PVOH/15TO@NZ hydrogels from 0 to 21 day are depicted in Table 4.

After 4 days of storage on the strawberries inside the uncoated box (blank sample) the growth of mold fungi is observed. In the case of strawberries inside the boxes coated with CS/PVOH and CS/PVOH/15NZ hydrogels the mold fungi is observed in 7th an 9th day correspondingly. On the contrary in the case of strawberries inside the PP box coated with the CS/PVOH/15TO@NZ hydrogels no mold fungi are observed until the 21st day of storage. So, it is obtained that CS/PVOH and CS/PVOH/15NZ hydrogels coated in the bottom side of PP boxes succeeded to protect the strawberries from mold fungi growth two and four days longer respectively than the uncoated blank PP box. Besides, the CS/PVOH/15TO@NZ active hydrogel succeeded to extent the protection of strawberries against the mold of fungi growth for three weeks. This result is very promising and shows that such CS/PVOH/15TO@NZ active hydrogel could be potentially applied as active pad inside the bottom side of fruit and vegetables boxes and extend their preservation time. It seems that such CS/PVOH/15TO@NZ active pad can protect fruit and vegetables due to its antibacterial properties and by releasing TO molecules inside the atmosphere of packaged fruit and vegetables as in the case of strawberries.

Table 4. Photos of packed strawberries inside the PP boxes uncoated (blank sample) and coated with CS/PVOH, CS/PVOH/15NZ and CS/PVOH/15TO@NZ hydrogels from 0 to 21 day.

	0 day	2nd day	4th day	6th day
BLANK				
CS/PVOH				
CS/PVOH/15NZ				
CS/PVOH/15TO@NZ				
	7th day	9th day	10th day	12th day
CS/PVOH				
CS/PVOH/15NZ				

CS/PVOH/15TO@NZ				
	14th day	**16th day**	**18th day**	**21st day**
CS/PVOH/15TO@NZ				

3. Conclusions

According to the results of the above-mentioned analytical methods, the biopolymeric matrix, originated by incorporation of the biodegradable byproduct chitosan and the biodegradable biopolymer poly-vinyl-alcohol, could be transformed to a promising food packaging material by addition of TO@NZ nanohybrid material. The overall study indicates that the addition of 15% TO@NZ nanohybrid material to CS/PVOH biopolymeric matrix led to a potential food packaging film with 34 % higher Young Modulus (E) and 46,5 % higher strength (σ_{uts}) compared to the relevant properties of the pure CS/PVOH film. The only measurable drawback was the elasticity of film which is represented by the elongation at break property. This property was reduced by -66%. All previous properties became worst while pure NZ added to the same CS/PVOH biopolymeric matrix. XRD and FTIR measurements indicated that the pure NZ could not be incorporated well with CS/PVOH biopolymeric matrix and creates aggregations inside the film. On the contrary, the same techniques indicated that the TO@NZ nanostructures are well incorporated with the CS/PVOH polymeric matrix and a homogeneous with no-aggregation phenomena film was developed. This achievement is due to the enforcement of dispersion offered by TO to the film.

As an overall conclusion is that the CS/PVOH/15TO@NZ film is the optimum developed material exhibits the higher antioxidant activity i.e., 53,4% higher than the relevant of the pure CS/PVOH, and the higher antimicrobial activity i.e., 134% for the *E. coli*, 87.5% for the *S. aureus*, 9% for the *S. enterica*, and 32% for the *L. monocytogenes*. The water-vapor diffusion coefficient and the oxygen permeability coefficient decreased down to -51% and -74% respectively, and thus the water-vapor and oxygen barrier increased. Such results are obvious by the in-vivo tests on strawberries, where in cases of CS/PVOH or CS/PVOH/xNZ, strawberries were rotted after 9 days while the strawberries packaged with CS/PVOH/15TO@NZ rotted after 21 days. This was resulted because of the modified atmosphere by the control-released TO.

4. Materials and Methods

4.1. Materials

Chitosan (CS) with a molecular weight of 100,000–300,000 was purchased from Acros-Organics company (Zeel West Zone 2, Janssen Pharmaceuticalan 3a, B2440, Geel, Belgium). Polyvinyl alcohol, 86-89% hydrolyzed, low molecular weight, was purschased from Thermo Scientific Chemicals Co., (168 Third Avenue, Waltham, MA USA 02451). Edible Activated Natural Zeolite was purchased by a local pharmacy market. The used Thyme Oil was produced by the Chemco company (Via Achille Grandi, 13–13/A, 42030 Vezzanosul, Crostolo, Italy) Hoffman Estates, IL, USA), Acetic acid (CAS Number: 64-19-7) was supplied by Sigma-Aldrich (Co., 3050 Spruce Street, St. Louis, MO, USA, 314-771-5765).

4.2. *Modification of NZ with thymol*

The modification of NZ with rich in thymol cluster took place according to previous reports [24,48]. Briefly, first thyme oil was distillated at 200 ^{o}C to remove the cluster of D-Limonene and p-Cymene. The remaining rich in thymol oil cluster (TO) was evaporated at 250 ^{o}C in a handmade apparatus in the upper part of which a 3 g of NZ bed has been adjusted. Differential Scanning Calorimetry showed that thymol was the main cluster of molecules adsorbed on NZ while the adsorption of TO molecules was rather physiosorbed than chemisorbed [25]. Thermogravimetric analysis experiments of NZ and TO@NZ showed that the wt.% amount of TO adsorbed was 35.5% [25]. The obtained TO@NZ nanohybrid was further stored to be used in the preparation of films.

4.3. *Preparation of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films*

First, an acetic acid (1% v/v) 2 wt.% CS aqueous solution was prepared by adding 20 g of CS powder in 990 mL of distilled water and 10 mL of glacial acetic acid. The solution heated at 70 ^{o}C and stirred overnight until a homogenous free of bubbles hydrogel was obtained. Second, 12 g of PVOH was added in 120 mL of distilled water, was stirred, and was heated since diluted to obtain a 10 wt.% PVOH aqueous solution. For each film 90 mL of acetic acid (1% v/v) 2 wt.% CS aqueous solution was mixed with 12 mL of 10 wt.% PVOH aqueous solution to obtain a 30 wt.% PVOH nominal content. In this CS/PVOH solution 0.21 g, 0.44 g and 0.71 g of NZ or TO@NZ powder was added to achieve 5, 10 and 15 wt.% NZ and TO@NZ final nominal content. The obtained mixtures homogenized at 18.000 rpm for 5 min to obtain CS/PVOH/xNZ and CS/PVOH/xTO@NZ hydrogels (where x=5, 10 and 15). The obtained hydrogels spread out on plastic petri dishes with 11 cm diameter and were let dried at 25 ^{o}C to evaporate water and obtain the final CS/PVOH/xNZ and CS/PVOH/xTO@NZ films. The obtained films piled of from petri dishes and stored at 25 ^{o}C and 50% RH for further use and characterization. For comparison a CS/PVOH film without the addition of NZ or TO@NZ powder was made and was considered as the "blank" sample. In Figure 6 images of all obtained CS/PVOH/xNZ and CS/PVOH/xTO@NZ films as well as pure CS/PVOH film are shown. In all cases high transparency was obtained and indicates a good dispersion of both pure NZ and TO@NZ nanohybrid in CS/PVOH/xNZ and CS/PVOH/xTO@NZ hydrogels.

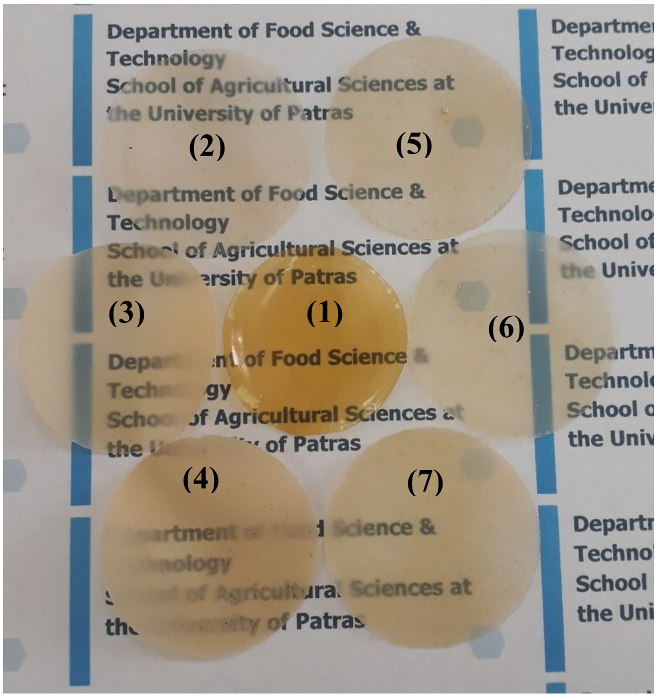


Figure 6. Photo of all obtained films. (1) pure CS/PVOH, (2) CS/PVOH/5NZ, (3) CS/PVOH/10NZ, (4) CS/PVOH/15NZ, (5) CS/PVOH/5TO@NZ, CS/PVOH/10TO@NZ and CS/PVOH/15TO@NZ.

4.4. XRD analysis of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

For XRD measuraments of each film an Brüker D8 Advance X-ray diffractometer instrument (Brüker, Analytical Instruments,S.A., Athens, Greece) was employed. A small piece of each film was attached to the sampler of the instrument and the measuraments carried out at in the range 2θ = 0.5–30° and with an increment of 0.03°.

4.5. FTIR spectroscopy of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

For the FTIR spectroscopy measurements an FT/IR-6000 JASCO Fourier transform spectrometer (JASCO, Interlab, S.A., Athens, Greece) was employed. Prior to measurements CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ films were catted in very small pieces and these small species incorporated to the KBr used for the measurements. The measurements were carried out in the range of 4000–400 cm^{-1} and with a 2 cm^{-1} resolution.

4.6. Tensile measurements of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

Tensile properties of obtained CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ films were carried out according to the ASTM D638 method by using a Simantzü AX-G 5kNt instrument (Simandzu Asteriadis, S.A., Athens, Greece).

4.7. Water Vapor Transmission Rate Measurements and Water Diffusion Coefficient Calculation

The Water Vapor Transmission Rate (WVTR $g/cm^2.s$) for all obtained CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ films, was measured at 38 °C and 95 %RH using a handmade apparatus and employing the ASTM E96/E 96M-05 method. The WVTR values were calculated and transformed to water vapor diffusivity (D_{wv}) values according to the theory and equations described in detail in previous publications [39,47].

4.8. Oxygen Transmission Rate Measurements and Oxygen Permeability Calculation

Oxygen Transmission Rate (OTR) values (cc $O_2/m^2/day$) for all obtained CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ films, were measured by employing an oxygen permeation analyzer (O.P.A., 8001, Systech Illinois Instruments Co., Johnsburg, IL, USA) at 23 °C and 0% RH according to the ASTM D 3985 method. The oxygen permeability coefficient values (PeO_2) were calculated according to the theory and equations provided in detail in previous publications [39,47].

4.9. Total antioxidant activity of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

The total antioxidant activity of all obtained CS/PVOH/xNZ and CS/PVOH/xTO@NZ films was evaluated according to diphenyl-1-picrylhydrazyl (DPPH) assay [37]. Briefly, 300 mg of each film were placed inside dark glass bottles with 10 mL of a 40 ppm ethanolic solution of DPPH. The absorbance at 517 nm wavelength of the DPPH solution was measured at 0 h and after 24 h incubation using a Jasco V-530 UV-vis spectrophotometer. For comparison the absorbance of a 10 mL of ethanolic DPPH solution without the addition of any film was measured at 517 nm and considered as the blank sample For each kind of film, three different samples were measured, and the statistical mean was achieved as the final measurement.

The % antioxidant activity after 24 h incubation of films was calculated according to the following equation:

$$\% \text{ Antioxidant activity} = (Abs_{blank} - Abs_{sample})/Abs_{blank} \times 100 \qquad (1)$$

4.10. Antibacterial Activity Tests of CS/PVOH/xNZ and CS/PVOH/xTO@NZ films

Antimicrobial activity of the films was investigated using the well diffusion method against four foodborne pathogenic bacteria. The bacteria tested included *Escherichia coli* (ATCC 25922) and *Salmonella enterica* subsp. *enterica* (DSMZ 17420), which are Gram-negative bacteria, as well as

Staphylococcus aureus (DSMZ 12463) and *Listeria monocytogenes* (DSMZ 27575), which are Gram-positive bacteria. These bacterial strains were obtained from the Institute of Technology of Agricultural Products, ELGO-DEMETER, located in Lykovryssi, Greece. Initially, the bacterial strains were cultured in Mueller Hinton Broth at a temperature of 37°C for a duration of 24 hours to allow for growth and achieve a bacterial concentration ranging from 10^7 to 10^8 colony-forming units per milliliter (CFU mL^{-1}). Following the overnight incubation, the bacteria were evenly distributed on Mueller-Hinton agar plates by rotating the plates at 60-degree intervals to ensure uniform growth of the bacterial colonies. Plates inoculated with the test organism had 6-mm wells cut into the surface of the agar using a cork borer dipped in alcohol and flamed. The wells were filled with 100 μL of the studied suspensions with which the final films were formed. The plates were then incubated at a temperature of 37°C overnight. After incubation, the diameters of any clear zones around the antimicrobial-containing wells were measured using calipers. This measurement was conducted to assess the extent of antimicrobial activity exhibited by the films against the tested bacteria. The entire experiment was performed in triplicate to ensure reliable and consistent results.

4.11. Packaging Test of CS/PVOH/HNT and CS/PVOH/TO@HNT based active pads in Strawberries protection against the fungi of mold

Considering the extensive research reported in the literature wherein CS/EO hydrogels were implemented as a protective coating for the preservation of strawberries, a novel methodology/technology for strawberry preservation was devised. This method was preconceived the most efficacious CS/PVOH/15TO@NZ sample as an active pad, positioned at the base of a PP plastic fruit container. The goal of this approach was the modification of the atmosphere inside the fruit container through the controlled release of TO from packaging material. In detail 50 mL of CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ solutions were spread in the bottom side of PP fruit boxes and had let them dry to make an active pad inside the PP box. Strawberries of as close to the same shape as possible and the same ripeness were purchased from the local supermarket they washed with water, and they were divided into four groups with three fruits each. In each box of PP coated with CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ solution three strawberries were putted, and the box was closed and kept under room temperature conditions (10-20 °C and 50% RH). For comparison three strawberries were putted inside an uncoated PP box which named as blank sample. The strawberries in the blank PP box as well as the strawberries inside the CS/PVOH, CS/PVOH/xNZ and CS/PVOH/xTO@NZ coated PP boxes were monitored with daily photos for 21 days to investigate possible mold of fungi growth.

4.12. Statistical analysis

Data were analyzed with the use of IBM SPSS Statistics 25.0. First, one-way analysis of variance (ANOVA) with Duncan's multiple range test was performed to compare mean values. All analyses were performed in triplicates.

Author Contributions: Synthesis experiment design, A.E.G., C.P. and C.E.S.; characterization measurements and interpretation, A.E.G., D.M., A.A, C. G., E.K., A.M.K., A. K., L. A., A. L., S,G., and C.E.S.; paper writing, A.E.G., C.P., E.K., and C.E.S.; overall evaluation of this work, A.E.G. and C.E.S.; experimental data analysis and interpretation, A.E.G., C.E.S., C. G., S,G., E. K., A.M.K., A.K., L.A., and C.P.; XRD, FTIR, OTR, tensile measurements, UV-vis spectroscopy, antioxidant activity, WVTR experimental measurements, and strawberries packaging test A.E.G., A.L., A.A, N.E.Z, S.G., and C.E.S.; SEM images D.M., A.A.; antimicrobial activity tests, E.K., A. K., L. A., and C.P. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: The datasets generated for this study are available on request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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