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DEVELOPMENT, CHARACTERIZATION AND APPLICATION OF CHITOSAN-BASED FORMULATION INCORPORATING *Crataegus orientalis* EXTRACT FOR FOOD CONSERVATION

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Abstract. In this investigation, chitosan-based films loaded with plant extracts from *Crataegus orientalis* (CR) were elaborated and evaluated in terms of structural, physicochemical and antimicrobial properties. Firstly, the CR extract was characterized by LC-MS/MS showing an abundance of Protocatechuic acid (56.82 µg/g) and Chlorogenic acid (67.13 µg/g). Then, the extract was incorporated into chitosan-based films at different concentrations (CHCR1-3). Findings revealed modifications in FTIR and XRD graphs as well as SEM micrographs following the incorporation of CR extract confirming the changes in the matrix structure and texture. Moreover, the addition of the extract reduced the transparency, swelling ratio, water solubility and moisture content potencies while increasing considerably the material thickness reaching 2.5-fold as regards CHCR3. Furthermore, Thermogravimetric findings showed three stages of degradation for CH control and CHCR2, with mass loss due to water evaporation and glycerol breakdown. Ultimately, CHCR bioformulations showed significant antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, especially in the case of the CHCR3, which was verified by the application as a preservative coating for lemon fruit. These encouraging results highlight the potential utilization of CHCR bioformulation as coating/packaging for perishable food products.

Keywords: *Chitosan film, Crataegus orientalis extract, antimicrobial.*

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1. Introduction

In the last decades, the harmful effect of synthetic packing materials on the environment and human health has been widely discussed (Khalil *et al*., 2016; Nurul Fazita *et al*., 2016). Consequently, the application of biodegradable and edible bio-based films as alternatives is becoming more required as a result of public awareness. Films developed from biocompatible and biodegradable biopolymers such as gelatin, alginate and chitosan showed the potential to replace petroleum-based plastics in several fields, including the biomedical and food industries (Díaz-Montes & Castro-Muñoz, 2021). Among the listed macromolecules, chitosan is favored owing to its notable properties,

including non-toxicity, biodegradability, biocompatibility, inherent antimicrobial activity and effective film-forming ability (Muxika *et al*., 2017). This biopolymer is obtained by the deacetylation of chitin. The latter is a naturally occurring and renewable biopolymer primarily extracted from marine and terrestrial sources e.g., crustaceans, fungi and insects (Borić *et al*., 2018), which confirms its availability in nature. As a result, chitosan-based films have emerged as a rival to the manufacture of edible films (Evcil & Karakaplan, 2022; Koc *et al*., 2020; Mouhoub *et al*., 2023a). Nonetheless, pure chitosan films present weak barrier properties and exhibit moderate bioactivities. Thus, the addition of bioactive compounds is necessary.

Numerous studies have focused on plant extracts as natural and bioactive additives due to their constituents being endowed with strong antioxidant and antimicrobial action (Bonilla & Sobral, 2016; de Almeida Soares & de Aquino Santana, 2024; Tural *et al*., 2025). Hawthorn, scientifically known as *Crataegus spp*., is a genus within the Rosaceae family, renowned for its extensive species range, estimated between 150 to 1200 (Christensen, 1992). These species manifest primarily as shrubs and trees, native not only to Turkey's flora but also to a wider geographical range (Edwards *et al*., 2012). In Turkey, all *Crataegus spp*. are colloquially known as ‘Alic,’ reflecting the local naming rather than indicating a specific genus complexity (Ercisli, 2004). These plants predominantly thrive in the lower and warmer forest margins and are ecologically significant, particularly in the context of their role in re-colonizing overgrazed pastures and abandoned farm fields (Yilmaz *et al*., 2010). *C. orientalis* (CR) has been traditionally employed in treating cardiovascular disorders, hypertension and atherosclerosis, for its richness in phenolic compounds and antioxidants (Coklar *et al*., 2018). Consequently, plant breeders are now focusing their efforts on cultivating crops like hawthorn (Çalişkan *et al*., 2012).

The chemical constituents of *Crataegus spp*. such as flavonoids, vitamin C, glycosides, anthocyanidins, saponins and tannins, contribute significantly to the plant's biological impacts, offering a range of bioactivities including neuroprotective, anticancer, antioxidant and antimicrobial effects (Nabavi *et al*., 2015).

In this context, the present work aims to incorporate *C. orientalis* extracts into chitosan films to develop a functional material intended for food conservation notably Mersin lemon which is a popular fruit in Türkiye due to its nutrients and minerals. However, its rapid ripening prevents the fruit from reaching export standards.

Several studies have investigated chitosan combined with bioactive compounds in food packaging and coating (Mouhoub *et al*., 2023b; 2024). However, the combination of chitosan and CR extract is original and has never been previously investigated as food-preservation material.

2. Material and methods

2.1. Solvents and reagents

Methanol, ethanol and acetic acid were acquired via Carlo Erba. Chitosan (CH), with low molecular weight and a deacetylation degree of 75% (CAS number: 9012-76-4, Sigma- Aldrich Product Number: 448869, Mw: 50-190 kDa), analytical quality phenolic standards, formic acid, ammonium formate and LC-grade acetonitrile and methanol were sourced from Sigma-Aldrich. All reactions were executed under an inert nitrogen atmosphere, utilizing a dry solvent. Pure water was obtained using the Milli Q water purification system (Millipore).

2.2. Extraction of Plant Material

Initially, the grinded and dried samples were introduced into the Soxhlet extractor. Subsequently, 200 mL of methanol was added to one flask and an equivalent volume of ethanol to the other. The extracts were then filtered using the Whatman filter paper. The ethanol and methanol extracts were then concentrated using a rotary evaporator to remove the solvents. Subsequently, the concentrated extracts were lyophilized (freeze-dried) to obtain the crude dry extracts. The resulting crude dry extracts were preserved in a deep freezer at -20°C for subsequent analyses (Aktepe *et al*., 2023).

2.3. The quantification of phenolic compounds using LC-MS/MS

Before LC-MS/MS analysis, all solutions were filtered via a Captiva Premium Syringe Filter, which has a nylon membrane (25 mm diameter and 0.45 μm pore size). The filter is encased in polypropylene. The analytical method was developed using all the phenolic chemicals that were available commercially. To prepare the 1.000 mg/L master stock solution, solid standard compounds were dissolved in methanol. Then, the solution was diluted using methanol to water (1:1). Ultimately, the prepared solutions were characterized using an Agilent 6430 triple quadrupole HPLC with mass spectroscopy (LC-MS/MS) (Aktepe *et al*., 2021). The chemical composition of CR extract is shown in Table 1.

2.4. Chitosan-based film preparation

In this study, we developed innovative films by combining chitosan and CR extract following casting methodology. The experimental design included four distinct formulations: pure chitosan control and three enriched films with different concentrations of CR extract.

The film preparation process began by dissolving chitosan powder (2% w/v) in an acetic acid solution (1% v/v) under continuous magnetic stirring at 200 rpm. For each film formulation, we utilized 200 mg of chitosan dissolved in 10 mL of the acetic acid solution. To enhance film flexibility, glycerol was incorporated as a plasticizing agent at a ratio of 150 μL per formulation (equivalent to 750 μL/g of chitosan) (Evcil, 2024). The experimental films, designated as CHCR1, CHCR2 and CHCR3, were prepared by incorporating 50, 75 and 100 mg of *C. orientalis* extract, respectively, while the control film did not contain the extract.

The homogenization process involved magnetic stirring of all solutions for 60 minutes at ambient temperature, followed by ultrasonic treatment using a WiseClean WUC A22H device at 40°C for 10 minutes to eliminate air bubbles and impurities. Subsequently, 3 mL aliquots of the homogenized solutions were carefully transferred to plastic molds (4 cm diameter) and allowed to dry for 48 hours at temperatures not exceeding 40°C to prevent thermal degradation. Upon completion of the drying process, the films were gently removed from their molds and stored either in desiccators at 25°C or in Petri dishes at 4°C for subsequent analytical procedures. The visual appearance of the prepared films is presented in Figure 1.

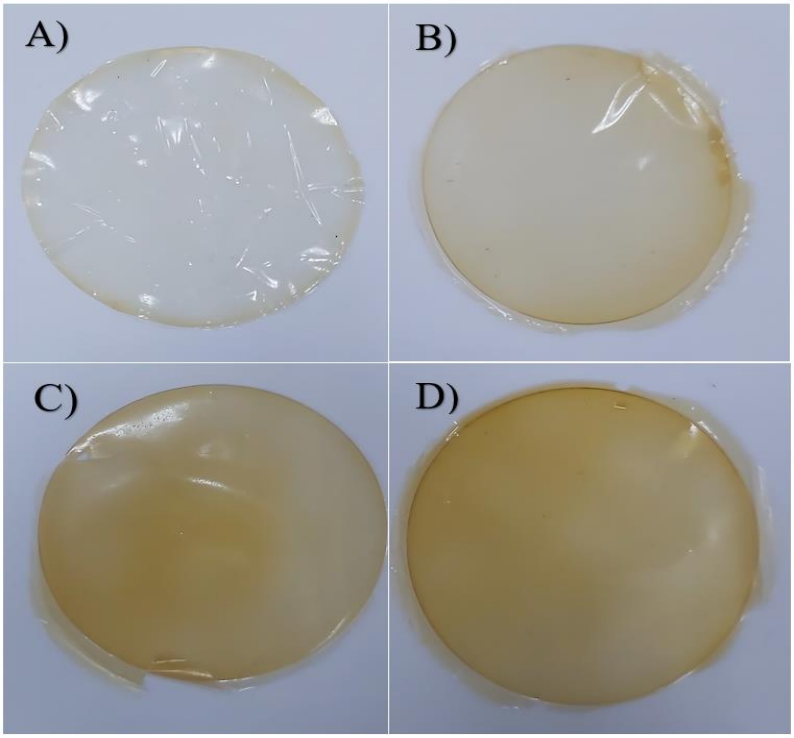

Figure 1. Images of (A) CH-Control film, (B) CHCR1 film, (C) CHCR2 film and (D) CHCR3 film

2.5. Film Characterization

2.5.1. Fourier-transform infrared spectroscopy analysis

The Fourier-transform infrared (FTIR) spectra of CH powder, plant extracts, CH-Control film and CHCR film were recorded at wavenumbers from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} using an Agilent 630. The measurements were conducted at ambient temperature.

2.5.2. X-ray diffraction (XRD)

The crystalline characteristics of CHCRs were assessed using Bruker-AXS-D8 Advance diffractometers with Ni-filtered Cu Kα radiation, scanning in the 2θ range from 0° to 60°. The X-ray analyses were carried out on CH powder, CH film and CHCR1-3 films.

2.5.3. Scanning electron microscopy (SEM)

The surface morphology of the film sample was examined with the LEO EVO 40 model SEM equipped with a secondary electron detector. Before examination, film samples were cut into small pieces and attached to double-sided conductive carbon tapes. Then, samples were covered with a 20 nm thick Au/Pd coating using a BAL-TEC SCD 050 sputter coater. The examination was performed at an acceleration voltage of 20 kV and under a vacuum pressure of 10^{-5} Pa.

2.6. Physical properties of films

2.6.1. Thickness of the Films

The thickness of the films was measured in millimeters (mm) using a digital micrometer (Insize, China). Measurements were taken at five distinct points for each film.

2.6.2. Moisture Content (MC)

Samples (2.4x2.4 cm^2) were initially weighed (M1) and subsequently dried at 105 °C. The moisture content was evaluated by measuring the weight reduction (M2) after 24 hours of drying at 105 °C. The moisture content was calculated as follows:

$$\textbf{Moisture content}: \left[\left(M1-M2\right)/M1\right]x100 \quad (1)$$

where M_1 is the initial weight of the film and M_2 is its final weight post-drying.

2.6.3. Solubility of Films in Water

The dried samples from the MC test were weighed (Wo) and then immersed in 6 mL of distilled water and continuously agitated for 24 hours at 25 °C. Next, the samples were dried again at 105 °C for 24 hours before the second weighing (Wf). The solubility was determined using the following equation:

$$\textbf{Water solubility (\%)} = \left[\left(\left(Wo-Wf\right)/Wo\right)x100\right] \quad (2)$$

where W_0 is the initial dry weight of the film and W_f is the final dry weight after immersion.

2.6.4. Swelling Ratio (SR)

Similarly to the water solubility determination, dried samples were weighed (W_0). Next, the films were placed in a beaker with 25 mL of distilled water and incubated for one hour. After the incubation period, the films were superficially dried with filter paper and then weighed (W). The following formula was used to determine the SR:

$$\textbf{Swelling Ratio} = \left[\left(\left(W-Wo\right)/Wo\right)x100\right]. \quad (3)$$

2.6.5. Optical transmittance

The optical characteristics of the CH films were defined by measuring the light transmittance using UV-VIS spectroscopy as suggested by Bajić et al. (2019). Briefly, the absorbance (A) of rectangular film samples was measured using a Lambda 40 UV/Vis spectrophotometer (Shimadzu-1900) throughout the wavelength range of 250-500 nm. The samples were placed in a cuvette for measurements with an empty cuvette as a reference. The results were reported as the samples' percent transmittance (%T), calculated as follows:

$$\%T = 10^{-A} \times 100\,. \quad (4)$$

2.6.6. Differential scanning calorimeter (DSC)

To examine the endothermic and exothermic characteristics of the CH (powder) and CHCR films, a DSC analysis was performed using a Shimadzu DSC-60. This analysis took place in a nitrogen atmosphere, spanning temperatures from -50 to 420 °C. Each test

used a 50 mg sample of the film, carefully sealed in a hermetic aluminum pan. The heating rate was controlled at 5 °C per minute to ensure consistent results.

2.7. Antimicrobial Properties

The disc diffusion method was used to examine the antimicrobial effects of the prepared films against the food pathogens Gram-negative *Escherichia coli* ATCC 25922 (*E. coli*) and Gram-positive *Staphylococcus aureus* ATCC 25923 (*S. aureus*) bacteria, as well as the fungus *Candida albicans* (*C. albicans*) (Hudzicki, 2012). Briefly, microorganisms were grown in appropriate media at optimum conditions. Merck's Mueller-Hinton agar was used as the medium to examine the antimicrobial effect. Solutions of each microorganism strain were prepared according to the McFarland 0.5 turbidity standard ($1.5x10^8$ CFU/mL) (Hudzicki, 2012; Zapata & Ramirez-Arcos, 2015). Sterile disks were impregnated with the CH solutions utilized for film elaboration and then placed on the inoculated medium. Next, the Petri dishes were kept at 37°C and 25°C as regards bacteria and fungus, respectively, for 24 hours. The inhibition zones were measured in mm using a digital caliper.

2.8. Coating of Lemon Fruit

Mersin lemons acquired from a local fruit vendor were firstly washed (0.01% NaCl aqueous solution for 2 minutes) and dried (ambient temperature). Then, the disinfected fruits were individually immersed in CH-Control film and CHCR2 solutions for 30 seconds, conducted in three replicates at 10-minute intervals. Uncoated fruits were considered as control. Next, the coated lemons were blow-dried at 30°C to form surface films and subsequently stored at ambient temperature for 12 days. Images of the control and coated lemons were taken on days 1, 8 and 12 (Nguyen *et al*., 2020).

2.9. Statistical analysis

All findings were tabulated as mean ± standard deviation (S.D). For paired or unpaired values, the Student t-test was performed. The p-value inferior to 0.05 is considered as significant.

3. Results and discussion

3.1. LC-MS/MS profile of CR extract

The phenolic profile of CR extract is presented in Table 1. Data revealed a dominance of chlorogenic and protocatechuic acids (> 50 μg/g), followed by isoquercitrin and vanillin (> 10 μg/g) and finally quercetin, o-coumaric acid, caffeic acid and hydroxybenzaldeyde (< 10 μg/g). The region where the plant is cultivated, along with factors such as color (yellow and red), drying conditions, solvent type, harvest time, extraction, and analysis procedures, have all been demonstrated in the literature to influence both the phenolic content and antimicrobial effect (Froehlicher *et al*., 2009; Liu *et al*., 2011; Šavikin *et al*., 2017; Coklar *et al*., 2018).

Phenolics containing hydroxyl and carbonyl groups can interact with the amino groups of chitosan, changing the physicochemical and biological properties of the films (Zawadzki & Kaczmarek, 2010).

Table 1. The chemical composition of CR extract

Compound	RT	*Crataegus Orientalis* (µg/g)
Protocatechuic acid	5.41	56.82
Chlorogenic acid	7.36	67.13
Hydroxybenzaldeyde	7.62	3.18
Caffeic Acid	7.84	6.97
Vanillin	8.61	12.28
o-coumaric acid	9.43	8.91
Isoquercitrin	11.74	19.47
Quercetin	14.88	9.73

***RT:** Retantion Time (min)

3.2. Analysis of Fourier-Transform Infrared Spectroscopy

The molecular interactions between the chitosan and the *C. orientalis* extract in the films have been established using FTIR analysis. Figure 2 displays the FT-IR spectra of the CHCR film, CR extract, chitosan and CH-Control film. OH bonds (3287 cm^{-1}), C-H aliphatic stretching vibration (2870 cm^{-1}), amide I (1651 cm^{-1}), amide II (1580 cm^{-1}), amide III (1375 cm^{-1}) and C-O stretching vibration (1021 cm^{-1}) were identified as distinctive chitosan peaks in the first spectrum.

The OH bonds (3280 cm^{-1}), C-H aliphatic stretching vibration (2870 cm^{-1}), amide I (1651 cm^{-1}), amide II (1561 cm^{-1}), amide III (1375 cm^{-1}) and C-O stretching vibration (1021 cm-1) were identified as the typical CH film peaks in the second spectrum. The CH film's spectrum demonstrates that structural alterations occurred during the film-formation process. The intermolecular interactions caused by the addition of glycerol to the film matrix (chitosan, acetic acid and water) expanded the OH bond and increased the intensity of the amide I, II and III bands.

The OH bonds (3280 cm^{-1}), aliphatic C-H stretching vibration (2922 cm^{-1}), aldehyde C-H stretching vibration (2855 cm^{-1}), ester C=O stretching vibration (1740 cm^{-1}), aromatic C-C stretching vibration (1617 cm^{-1}) and C-O (1233 and 1021 cm^{-1}) were found to be characteristic peaks of the CR extract.

The CHCR film spectrum shows bands at 2109 and 1408 cm^{-1}, which correspond to the benzene skeleton vibration and the C-H stretching vibration of the benzene ring, respectively. A higher intensity of absorption was observed for amide II (1561 cm^{-1}) and amide III (1371 cm^{-1}) as a result of molecular interactions between the film matrix and CR extract. The spectrum of CHCR film demonstrates that all of the distinct peaks of the extract and CH film peaks namely emerged at 1640 and 1740 cm^{-1}.

The addition of CR extract to the chitosan resulted in the observation of additional peaks in the current investigation at 1640 and 1740 cm^{-1}. The imine bond between hydroxybenzaldehyde and vanillin in the CR extract and chitosan is indicated by the peak at 1640 cm^{-1}. According to Mayachiew and Devahastin (2010) a new peak at 1740 cm^{-1} was identified and explained as the result of an ester bond that occurred between polyphenolic chemicals and chitosan. This result was consistent with the findings of Mayachiew and Devahastin. The results of the FTIR study showed that CR extract was successfully incorporated into the chitosan film, leading to different molecular interactions in the film matrix.

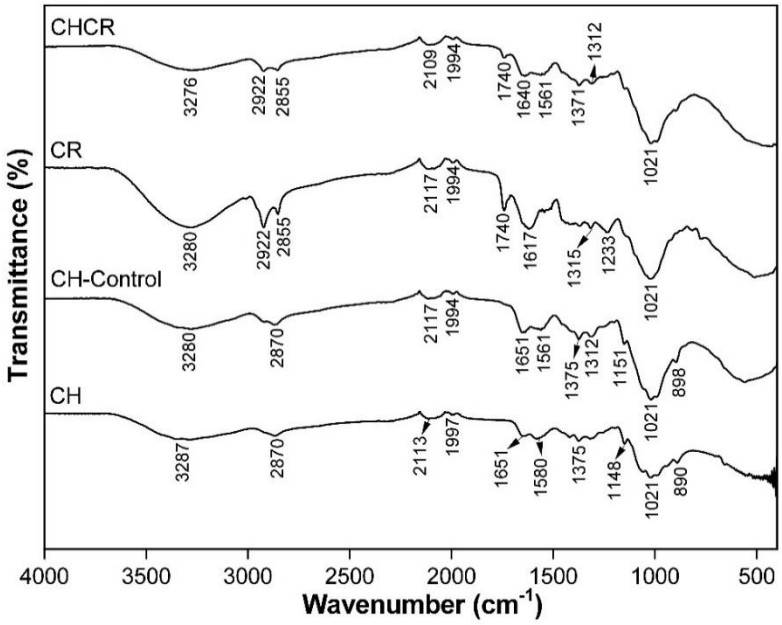

Figure 2. IR Spectra of CH (powder), CR extract and both CH-Control and CHCR films

3.3. XRD patterns of CHCRs

A valuable insight into the structure of chitosan films is provided by X-ray diffraction (XRD) patterns (Figure 3). CH has a semi-crystalline structure with two main peaks at around 2θ=14° and 20°, indicating inter- and intra-molecular hydrogen bonds in the presence of free amino groups. The XRD pattern seen in Figure 3 is indicative of the crystalline properties of both CH and CHCR films. Around 20°, the XRD peak corresponds to the normal fingerprint of CH-Control film. Similar results were reported previously (Liu *et al*., 2017b). The addition of CR extract at different concentrations to the CH film results in a significant alteration in the crystallinity of the composite film ($p < 0.05$).

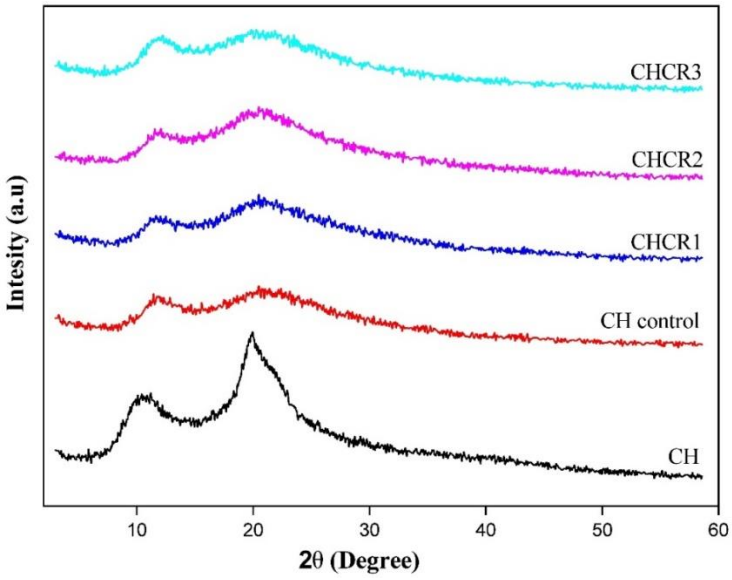

Figure 3. XRD of CH(Powder) and both CH-Control and CHCR films

The CHCR film becomes less crystalline with an increase in CR content and a minor diffraction peak disappears, indicating a structural change in the film from crystallite to amorphous. Similar findings were reported by Sun et al. (2017) where the CH-Control film was supplemented with young apple polyphenol, indicating that the interaction between polyphenols and CH influences intra- and inter-hydrogen bonding in the CH-Control film, resulting in a decrease in the film's crystallinity. Furthermore, the type and amount of phenolic material in the film result in different crystalline peak intensities (Kadam & Lele, 2018; Liu *et al*., 2016).

3.4. Scanning Electron Microscopy Analysis

SEM images were used to examine the surface morphologies of CHCR films (Figure 4). CHCR films showed a rough and heterogeneous surface. This could be attributed to the heterogeneous nature of CR extract. The CHCR1 and CHCR2 films showed a less rough surface compared to CHCR3 due to the higher grafting ratio of the CR extract in the latter. According to the results, the CR extract ratio influences the appearance of the CHCR film, which is most likely caused by hydrogen bonding. This result is supported by a previous report (Liu *et al*., 2017a).

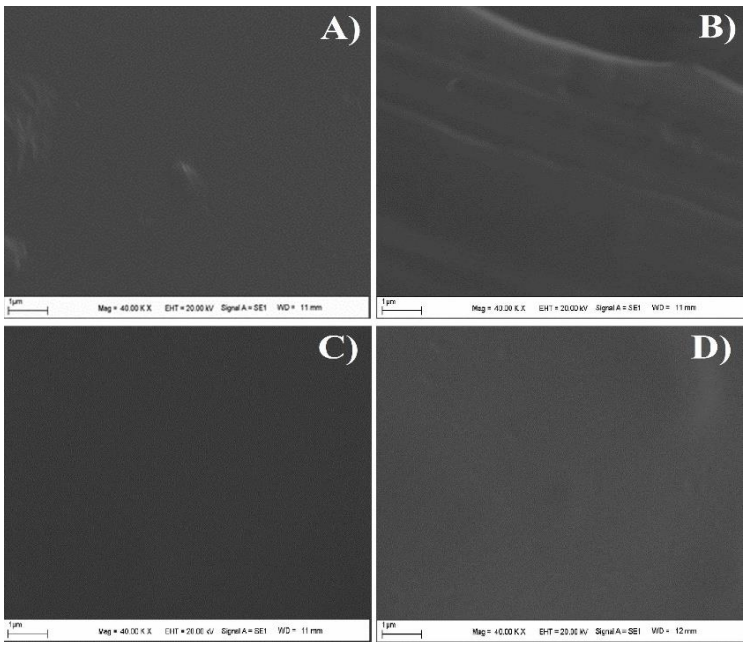

Figure 4. SEM Micrographs of A) CH-Control, B) CHCR1, C) CHCR2, and D) CHCR3 films

3.5. Thermal properties of CH and CHCR films

Figure 5 illustrates the TGA thermograms of CH (powder) and CHCR films. Three stages of degradation were observed for both films. The first stage (between 25 and 210 °C) is attributed to the evaporation of water from the polymeric structures. The second phase, is associated with the breakdown of glycerol, which occurs at a temperature between 200 and 430°C (Dou *et al*., 2009). The denaturation of plant extracts and the chitosan polymeric structure were the primary sources of the mass loss, which was observed between 438 and 664°C (Zawadzki & Kaczmarek, 2010). Ash contents for the CH and CHCR films were measured following the thermal degradation and they were

16.58% and 23.38%, respectively. The addition of plant extracts generally had no effect on the thermal stability.

Figure 5 displays also the DSC thermograms of CH and CHCR films. One exothermic peak (447.28°C) and two endothermic peaks (36.03 and 234.28°C) were visible in the case of CH film. The phase inversion temperature of the chitosan breakdown process was represented by the first exothermic peak. Peng et al. (2013) reported similar results. Additionally, they clarified this phase inversion temperature was lowered due to the incorporation of the plant extract. The current study produced comparable findings. With the addition of the CR extract, the crystallinity of the film decreased resulting in a drop of thermal stability (Martins *et al*., 2012). CHCR2 film showed two endothermic peaks (27.29 and 209.55°C). The evaporation of solvent traces (water, acetic acid and methanol) utilized in the creation of both films is responsible for the first endothermic peaks (Altiok *et al*., 2010). However, the denaturation of the extract and glycerol components is responsible for the second endothermic DSC signal observed in the CHCR2 film. The Tg value shifted from 234.28 to 209.55 °C upon the incorporation of CR extract. This phenomenon can be explained by the plasticizing effect of the extract. The plasticizer induced the reorganization of the polymer chains inside the film matrix through the interaction between the plasticizer and the polymer. Consequently, it often lowers the Tg value of the polymer due to enhanced chain mobility. The reduction in the Tg value in this study is ascribed to the enhanced mobility of the polymer chains within the CHCR2 film. The elasticity and extensibility of the CHCR2 film were superior to those of the CH-control film (Kaya *et al*., 2018).

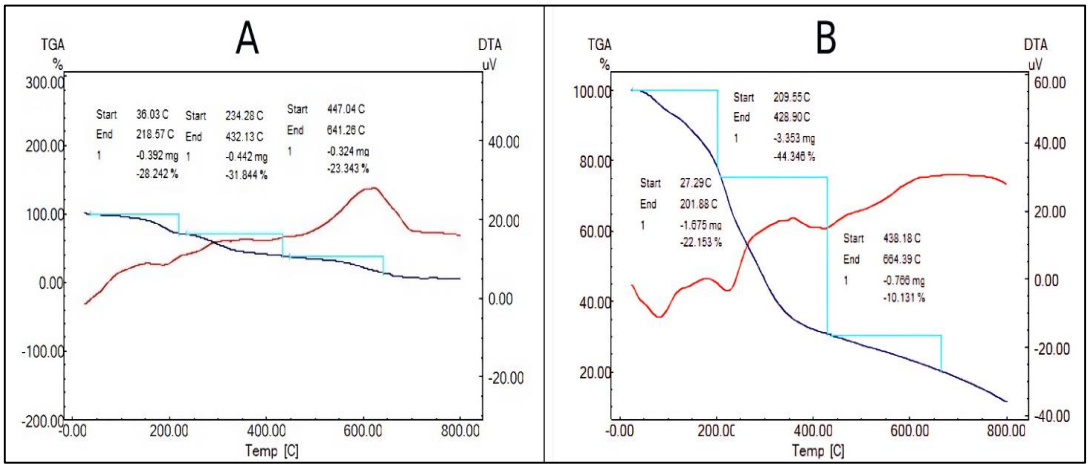

Figure 5. DSC (red) and TGA (blue) thermograms of A) CH-Control B) CHCR2 films

3.6. Water Solubility and Thickness

According to Table 2, a slight decrease in the water solubility was noticed following the addition of CR extract. Analogous findings were reported as regards the water solubility rate of the films (Rambabu *et al*., 2019). The availability of the amino and hydroxyl groups of the polymer was reduced by the strong interaction with the phenolic compounds of CR extract (Hafsa *et al*., 2016). As a result, the CHCR films became less soluble in water.

The CH, CHCR1, CHCR2 and CHCR3 films present thicknesses of 0.051 mm, 0.076 mm, 0.101 mm and 0.127 mm, respectively. The film thickness increased significantly with the increase of the concentration of CR extract. This might be related

to the hydrogen bonds between the phenolic compounds and the chitosan polymer chains. These bonds facilitate the formation of a denser and more robust chitosan matrix (Sun *et al*., 2017).

3.7. Moisture Content (MC%) and Swelling Ratio(SR)

Similar to the water solubility, the MC of the film decreased moderately with the increase of CR concentration ($p < 0.05$). The interactions between chitosan and CR extract may reduce the availability of hydroxyl and amino groups, which minimize the chitosan-water interactions through hydrogen bonding (Wang *et al*., 2019). This MC variation was confirmed by the drop in SR. The lower the swelling ratio of a polymeric film, the higher its water resistance. In packaging applications, a decrease in water absorption through sheets is typically preferred. Basically, the ratio and type of intermolecular interactions in polymer chains have been shown to significantly influence the swelling ratio (Nguyen *et al*., 2020). In the case of the CHR films, fewer polar groups were available to interact with water molecules, resulting in a decreased swelling ratio, which indicates that the functional groups of the CR extract components interacted with the polar groups of the polymer (Wang *et al*., 2019; Uranga *et al*., 2019).

Table 2. Thickness, moisture content and water solubility of films

Samples	Thickness (mm)	Water Solubility (%)	Moisture Content (%)	Swelling Ratio (%)
CH film	0.051 ± 0.03^{d}	28.1 ± 0.84^{a}	21.2 ± 0.53^{a}	40 ± 0.90^{a}
CHCR1	0.076 ± 0.04^{c}	27.5 ± 0.78^{a}	20.3 ± 0.48^{ab}	32 ± 0.82^{b}
CHCR2	0.101 ± 0.05^{b}	26.9 ± 0.56^{ab}	20.1 ± 0.62^{ab}	29 ± 0.74^{c}
CHCR3	0.127 ± 0.08^{a}	25.9 ± 0.72^{b}	19.5 ± 0.58^{b}	25 ± 0.74^{d}

3.8. Optical transmittance

Transparency is a crucial property in identifying the range of applications for developed films. The visual attributes of chitosan-based films, such as glossiness and transparency, may significantly influence customer acceptability (Vilela *et al*., 2017). When considering the photostability of particular food ingredients, the barrier property against harmful light is a required parameter (Duncan & Chang, 2012). The light transmission curves for CH-Control and CHCR films are shown in Figure 6.

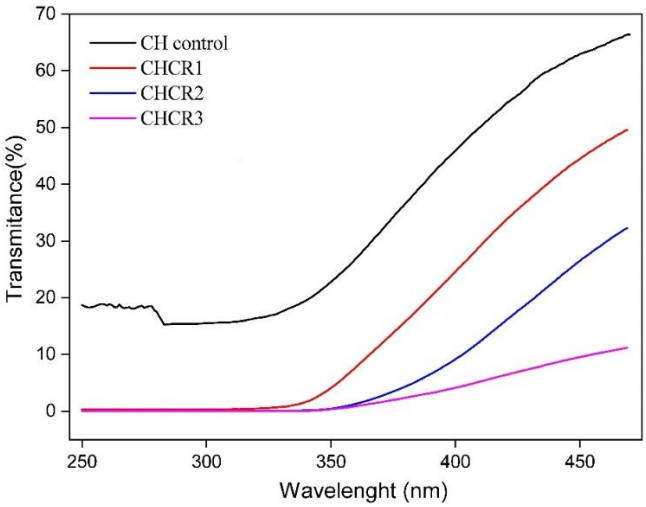

Figure 6. The light transmittance of CH-Control and CHCRs in the UV–vis light region

Compared to CHCR films, the light transmittance of CH-Control film was significantly higher. The transparency and the concentration of the added CR extract are inversely correlated. At 500 nm, the transmittance rates of CH, CHCR1, CHCR2 and CHCR3 films were 66.37%, 49.54%, 32.28% and 11.16%, respectively. The presence of pigments and tints on the film surface may be the cause of the blend films' progressive decline in transparency rate.

3.9. Antimicrobial Properties

The antimicrobial effects of the CH-Control and CHCR films were examined on food-related pathogens using the disc diffusion method (Figure 7). Findings showed that the inhibition zones exhibited by the CHCR films were significantly larger compared to CH-Control film, especially in the case of CHCR3 film (Table 3), where the inhibition zone diameters were equal to 12.41, 13.13 and 14.37 mm as regards *E. coli*, *C. albicans* and *S. aureus*, respectively. Plant extracts are rich in phenols and flavonoids. These compounds exhibit bioactive properties, including antimicrobial and antioxidant activities (Rauf *et al*., 2019). The LC-MS/MS profile shows high concentrations of phenolic and flavonoid compounds, including protocatechuic acid, chlorogenic acid, vanillin and isoquercitrin. The presence of these molecules enhanced the antimicrobial activity of the film (Eroglu & Girgin, 2021; Seal *et al*., 2017). Phenolic and flavonoid compounds found in plant extracts increase the ROS (reactive oxygen species) level in microorganisms (Tungmunnithum *et al*., 2018). In addition, these secondary metabolites cause structural and functional disruption of cell membranes as well as the inhibition of important enzymes such as DNA gyrase, protein kinase and dehydratase (Rempe *et al*., 2017; Yang *et al*., 2022).

Based on Table 1, the components with the highest proportions in the CR extract were protocatechuic acid and chlorogenic acid whose antimicrobial action was widely studied (Kabir *et al*., 2014; Chai *et al*., 2019).

Table 3. Inhibition zones generated by CH and CHCR films against *E. coli*, *C. albicans* and *S. aureus*

Microorganisms	Inhibition zone diameter (mm)			
	CH-Control film	CHCR1	CHCR2	CHCR3
E. coli	0.84±0.04^{d}	3.39±0.21^{c}	9.85±0.43^{b}	12.41±0.25^{a}
S. aureus	0.70±0.05^{d}	5.01±0.17^{c}	7.49±0.05^{b}	14.37±0.24^{a}
C. albicans	1.04±0.09^{d}	7.49±0.13^{b}	5.01±0.52^{c}	13.13±0.11^{a}

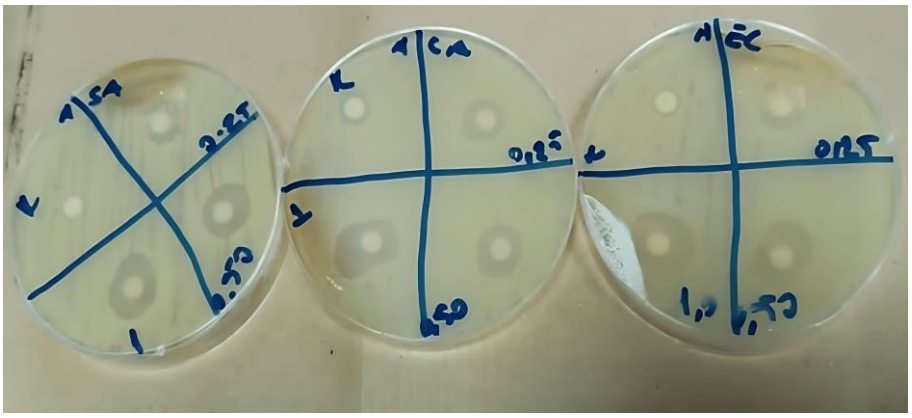

Figure 7. Images showing the inhibition zones generated by CH and CHCR films against *E. coli*, *C. albicans* and *S. aureus*

3.10. Food Preservation Application

According to Figure 8, the outer skin of all lemons was fresh yellow on the first day of storage. The uniform yellow color of lemons changed to brownish-yellow with spots and freckles after eight days, indicating decay. The general appearance of the lemon coated with CHCR film was better compared to the one coated with CH-Control film and the control fruit. Moreover, the uncoated lemon and CH control-coated lemon shrank due to water loss, while the CHCR-coated lemon remained in a good state even at the end of day 12, confirming the water barrier property of the CHCR coating. This demonstrated that incorporating CR into chitosan coating can mitigate the decay of lemons. Further research is needed to evaluate sensory properties, texture and nutritional content (Jongsri *et al*., 2016; Shiekh *et al*., 2013).

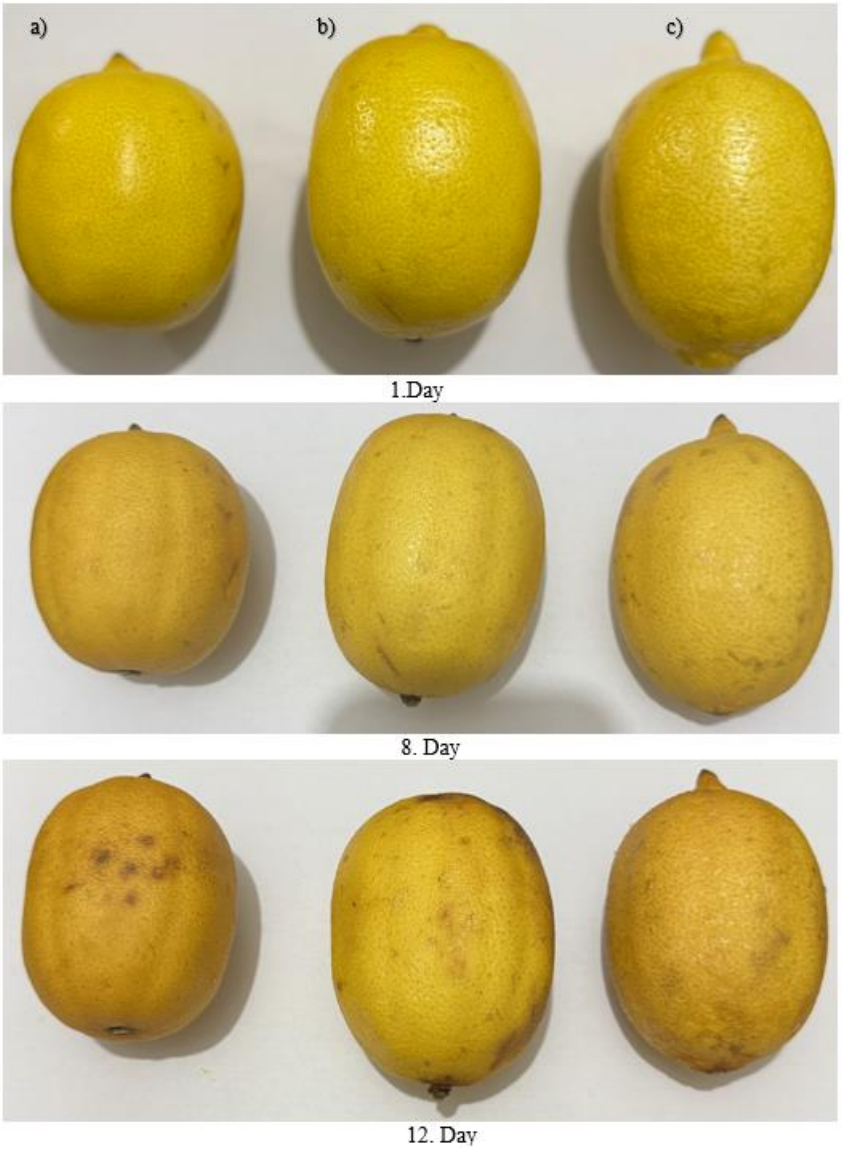

Figure 8. Evolution of a) uncoated lemon and b) lemon coated using CH-Control and c) CHCR2 coating

4. Conclusion

In this study, we successfully incorporated CR extract (rich in Protocatechuic and Chlorogenic acids) into chitosan films and evaluated the properties and biological activities of the prepared bioformulation. The addition of extract modified the structure and texture of the material while improving its opacity, water resistance and barrier and antimicrobial activity against *E. coli*, *C. albicans* and *S. aureus*. This improvement in bioactivities and water barrier characteristics was verified in the lemon preservation test, where the fruits coated with CHCR showed a good appearance compared to the lemons coated with CH-Controlonly and the uncoated ones. Based on these promising results, we claim that CHCR bioformulation could potentially be used for food conservation purposes. However, optimization of extraction conditions and investigation of the suitability of the films for industrial production is recommended for future studies. Further investigation on industrial scalability and consumer acceptance is recommended.

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