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RESEARCH ARTICLE

Molecular guardians: Unveiling chitosan film defense through analytical microscopy

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Abstract

In response to growing environmental concerns about plastic packaging, this study examines biopolymer-based alternatives, focusing on the development and performance of chitosan-based films enhanced with polyvinyl alcohol (PVA), polyethene glycol (PEG), glycerol and essential oils as eco-friendly packaging solutions. Using the solvent casting method, chitosan films were prepared with incorporated essential oils to enhance their functional characteristics. FTIR analysis confirmed the successful fabrication of the films by identifying functional groups such as -OH, N-H, C=O, C≡N, C-Cl, C-Br and metal-O bonds. SEM analysis revealed smooth surfaces with minimal residues, indicating partial uniformity. Antimicrobial testing revealed that sample S_1 exhibited concentration-dependent inhibition, whereas sample S_2 demonstrated strong, broad-spectrum activity against *Staphylococcus aureus* and *Escherichia coli* at higher concentrations. Antioxidant activity, evaluated using the DPPH assay, revealed that at 10 µL of sample S_2, 76.22 % inhibition was achieved and sample S_1 showed 75.40 %, with effectiveness declining at higher concentrations. The findings underscore the promise of chitosan-essential oil films as multifunctional packaging materials with both antibacterial and antioxidant properties. In horticultural applications, these biofilms present a sustainable alternative to traditional plastics, contributing to improved product quality and extended shelf life during postharvest processes and marketing.

Keywords: antioxidant; antibacterial; chitosan; packaging; sustainable

Introduction

Biopolymers are being investigated as environmentally beneficial alternatives to plastic packaging. Biopolymers are non -toxic, biodegradable and help reduce plastic waste because they are made from renewable resources such as chitosan and starch. Their antibacterial and moisture-regulating qualities improve product preservation. Packaging affects purchases and how customers perceive a product. Profitability may be lowered by losses resulting from improper harvesting, handling and storage. Materials need to be easy to handle, water-resistant, economical or recyclable and long-lasting for transportation. Cellophane, cardboard, butter paper and other polymers are common choices. Through the preservation of structural, biological and pathological integrity throughout the marketing process, effective packaging maintains the quality of flowers (1). Packaging films are thin layers of edible polymers that act as active barriers in food packaging, making handling simple. They control the exchange of gases, moisture and scents, unlike glass or metal and are impacted by the characteristics and design of the material (2). Although petroleum-based polymers, such as polyethene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethene terephthalate (PET) and polyamide (PA), are widely used in packaging, their non-biodegradability results in significant pollution. Chitosan (CH), chitin (CT), collagen (COL), sodium alginate (SA), agar (AG) and carrageenan (CR) are examples of biopolymers made from marine biomass that provide sustainable substitutes for these materials (3).

The use of bio-based packaging, particularly chitosan, to prolong the shelf life of perishables is becoming more popular because of its antibacterial, biodegradable, biocompatible, non-toxic, excellent film-forming ability, high reactivity and chemical stability qualities. While combining with alginate and polyvinyl alcohol (PVA) increases strength and flexibility, infusing essential oils improves preservation. Chitosan-based films are perfect for food, flower preservation, agriculture and medicine since they are more durable when natural fibers are added (4).

Chitosan, a natural polymer, is a sustainable alternative to polypropylene, extending the shelf life of fruits, vegetables and flowers through film packaging and coatings. Like chitin, chitosan is a non-aromatic solid that looks white to light yellow or grey flakes or powder and is insoluble in water. Acidic solutions (pH < 6.3), such as those containing lactic, citric, formic, acetic, malic, or tartaric acid,an dissolve it and produce films (5). Researchers enhanced biodegradable chitosan films by

incorporating plasticizers, blending films with different molecular weights of polyvinyl alcohol and maleic acid to improve their strength. The chitosan-polyvinyl alcohol combination shows strong potential for sustainable packaging (6).

Essential oils are concentrated, hydrophobic liquids containing volatile compounds that define a plant's fragrance. Extracted from various parts, they exhibit antibacterial, antioxidant, antiviral and insecticidal properties. Used in cancer treatment, food preservation, aromatherapy, perfu mery, cosmetics, beverages, textiles and pharmaceuticals, they also offer hepatoprotective benefits, varying by chemotype. Essential oils are gaining attention as natural additives for extending the shelf life of food products, film packaging and nano emulsions, offering a safer alternative to synthetic preservatives and packaging. Their antioxidant and antimicrobial properties help reduce food spoilage (7). Essential oils in packaging protect food from pathogens and spoilage, serving as an active form of packaging. However, their volatility can cause strong odors, limiting use at high concentrations, while lower levels maintain acceptability (8).

Materials and methods

Chitosan-based films were prepared using the solvent casting method, with chitosan (Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India) as the primary biopolymer and Poly (vinyl alcohol) (PVA, 99 % hydrolyzed; Sigma Aldrich, Bengaluru, India) as the secondary biopolymer in the film formulation. Polyethylene glycol (PEG, molecular weight 6000; HiMedia Laboratories, Karnataka, India) and D-sorbitol (MW: 181.17; CDH Fine Chemicals, New Delhi, India) served as the main plasticizers, while glycerol (molecular weight 92.09; CDH Fine Chemicals, New Delhi, India) was used to enhance the flexibility of the film. Essential oils were obtained from the Department of Plantation and Spices at Tamil Nadu Agricultural University. They were incorporated as separate components.

Sample preparation

A chitosan-glycerol film without essential oil was prepared by dissolving 2 g of chitosan in 2 % acetic acid with continuous stirring. Separately, 1 g of PVA is dissolved in 100 mL of water by heating to 80-90 °C for 1 hr, then cooled. PEG and sorbitol, each weighing 1.5 g, were dissolved separately in 100 mL of water and then combined. The PEG-sorbitol solution is added to the chitosan solution while stirring at 1200-1500 rpm, followed by 3 mL of glycerol and the PVA solution and the mixture is stirred for 6 hr. After 10 min of rest, the solution is poured into molds, dried for 3 to 4 days, peeled, acclimated and stored.

A chitosan-glycerol film with essential oil was prepared by dissolving 2 g of chitosan in 2 % acetic acid, with continuous stirring. Separately, 1 g PVA was dissolved in 100 mL of water by heating to 80-90 °C for 1 hr, then cooled. 1.5 g of PEG and 1.5 g of sorbitol were each dissolved in 100 mL of water and then combined. The PEG-sorbitol solution was added to the chitosan solution while stirring at 1200-1500 rpm, followed by 3 mL of glycerol, 2 mL of clove bud essential oil and the PVA solution. The mixture was then stirred for 6 hr. After stirring, the solution was allowed to rest for 10 min to acclimate to room temperature and was then stored before being poured into moulds. It was spread evenly and left to dry in a naturally ventilated room for 3 to 4 days. The required quantity of solution varies based on the mould size, with 60 mL needed for a 6-inch mould and 70 mL for a 7-inch mould. Once dried, the film was peeled off, allowed to acclimate to room temperature and stored.

Sample 1: Chitosan- film (without essential oil)

Sample 2: Chitosan- film (with essential oil)

Characterization of sample

Fourier transform infrared spectroscopy (FTIR)

A Jasco FT-IR-6800 Spectrometer was used to examine the molecular interactions and structural alterations in chitosan films with and without essential oils. To ensure precise characterization, films were placed on a NaCl crystal holder and spectra were captured from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} and a scanning speed of 2 mm/s, without zero filling.

Scanning electron microscopy (SEM)

The surface morphology of chitosan films was analyzed both with and without the incorporation of essential oils using a Scanning Electron Microscope (SEM) (TESCAN MIRA3 XMU). SEM micrographs were created at different magnifications.

Antimicrobial activity

Growth and inoculation

The growth method used was the Kirby-Bauer method. This method is superior to the MIC (Minimum Inhibitory Concentration) or MBC (Minimum Bactericidal Concentration) method, as it is more suitable for preliminary, rapid and comparative assessment of antimicrobial activity, especially when working with biofilms. Firstly, five isolated colonies of an agar plate culture were set. A loop was used to transfer the growth into a tube containing 4 to 5 mL of the broth medium (Muller-Hinton broth), which was then incubated at 35°C until the desired turbidity was reached. The turbidity of the actively growing broth culture was then adjusted to achieve a concentration of approximately 1 to 2 × 10^8 CFU/mL for *Staphylococcus aureus* and *E. coli*. For inoculation, a sterile cotton swab was dipped into the adjusted suspension and pressed against the inside wall of the tube to remove the excess inoculum. The dried surface of a Muller-Hinton agar plate was inoculated by streaking the swab evenly across the sterile agar in three directions, ensuring uniform distribution. The plate was left to absorb excess moisture for 3 to 5 min before placing drug-impregnated disks. A well of 6 mm in diameter was then made in the agar and filled with 10 to 50 µL of a standard antibiotic (gentamicin) and the sample. Those plates were incubated at 37 °C for 24 hr for complete diffusion in an inverted position.

Antioxidant activity

The ability of films to scavenge radicals against 0.1 mM DPPH (2,2 -diphenyl-1-picrylhydrazyl) in methanol was measured using the DPPH assay by Blois in 1958 to assess antioxidant activity. Absorbance at 517 nm was measured following a 30 min dark incubation period and inhibition (%) was computed using ascorbic acid as the reference.

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{A control-A sample})}{\text{A control}} \times 100 \quad \text{(Eqn. 1)}$$

Results and Discussions

Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis of sample 1 revealed a faint peak at 3857 cm^{-1} that indicated free O-H groups and a broad band at 3340 cm^{-1} for overlapping O-H and N-H stretching. A large peak confirmed C-O stretching at 1041 cm^{-1} and a moderate contribution from C-N is due to the deacetylation of chitin. Peaks located at 725, 648 and 601 cm^{-1} were associated with C-Cl stretching, C-Br stretching and CH_2 rocking, respectively. The presence of O indicated metal complexes-O and metal-O vibrations at 447, 470 and 515 cm^{-1} as shown in (Fig. 1). Sample 2 showed weak C-H and N-H stretching at 3109.25 cm^{-1}, indicating aromatic/alkene groups, whereas O-H stretching was visible at 3865.55 cm^{-1}. An indication of CO_2 or C⊠N stretching was a dip at 2330.01 cm^{-1}. There was confirmation of CH_2 bending, C-Cl stretching and metal-O interactions by peaks at 725.32, 686.66 and 648.08 cm^{-1}. Strong bands signalled skeletal and metal-O vibrations at 555.50, 509.21 and 455.20 cm^{-1} (Fig. 2).

Scanning electron microscopy (SEM)

The scanning electron microscopy of all the films showed similar morphological characteristics. The SEM analysis reveals a smooth texture along with a few solid substances, which suggests an incomplete homogenization of the blends (Fig. 3 & 4).

Antimicrobial activity

The antimicrobial activity of *Staphylococcus aureus* and *Escherichia coli* was evaluated using the zone of inhibition (mm). The results indicated that sample 1 showed a concentration-dependent antimicrobial effect likely due to insufficient active compounds at lower concentrations, with no inhibition at 10 µL, moderate zones at 20 µL (15 mm for *S. aureus*, 10 mm for *E. coli*) and slightly larger zones at 30 µL (16 mm and 12 mm, respectively), indicating greater efficacy against gram-positive bacteria (Fig. 5). At higher concentrations, improved diffusion and membrane disruption resulted in increased inhibition zones, indicating enhanced antibacterial efficacy. In contrast, sample 2 displayed a threshold effect, showing no activity at lower concentrations but strong, equal inhibition (20 mm) against both strains at 30 µL (Fig. 6). This suggests that essential oil components, such as phenols and terpenes, must reach a minimum concentration to disrupt bacterial membranes and exert broad-spectrum activity. In a related study, chitosan/silver nanoparticle films were created using a simple, eco-friendly photochemical process. The films demonstrated potent antibacterial activity against *Escherichia coli* and *Bacillus*, indicating their potential for application as antimicrobial coatings on packaging materials, wound dressings and medical implants (9)

Antioxidant activity

The antioxidant activity of two distinct samples (S_1 and S_2) was evaluated using the DPPH free radical scavenging assay, employing ascorbic acid as the standard reference. A regression coefficient (R^2)of 0.9884 demonstrated the standard's high degree of reliability and at a concentration of 15 µg/mL, it achieved a maximum per cent inhibition of 95.90 %, indicating a strong positive association between concentration and antioxidant activity (Table 1). Sample 2 had the highest antioxidant potential of all the examined samples at 10 µL, with a peak inhibition of 76.22 %. As concentrations increased, the activity gradually declined (Table 2). Significant free radical scavenging ability was also demonstrated by sample 1, which attained a maximum inhibition of 75.40 % at the same starting concentration and then declined similarly as the concentration raised (Table 3 & Fig. 7). In support of their potential as efficient antioxidant food packaging materials, a prior study showed that chitosan-protocatechuic acid (PA) composite films exhibited significantly increased antioxidant activity, with a higher release of total phenolic content into both aqueous and fatty food simulants (10).

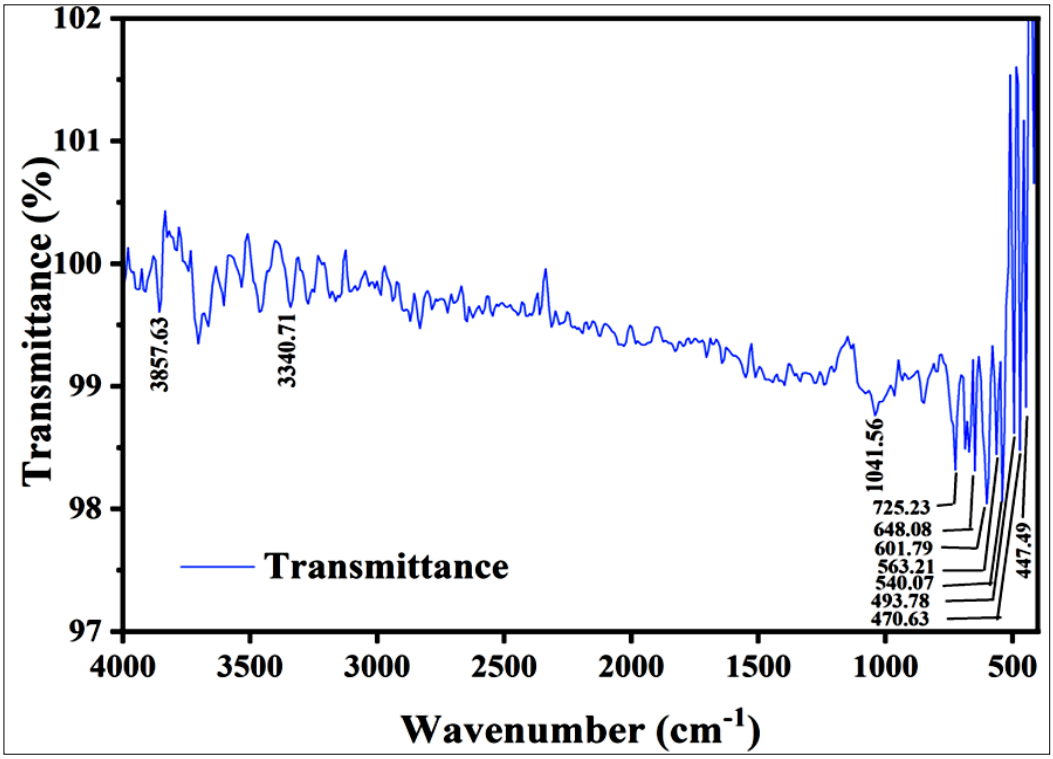

Fig. 1. FTIR spectra of sample 1 (chitosan film without essential oil).

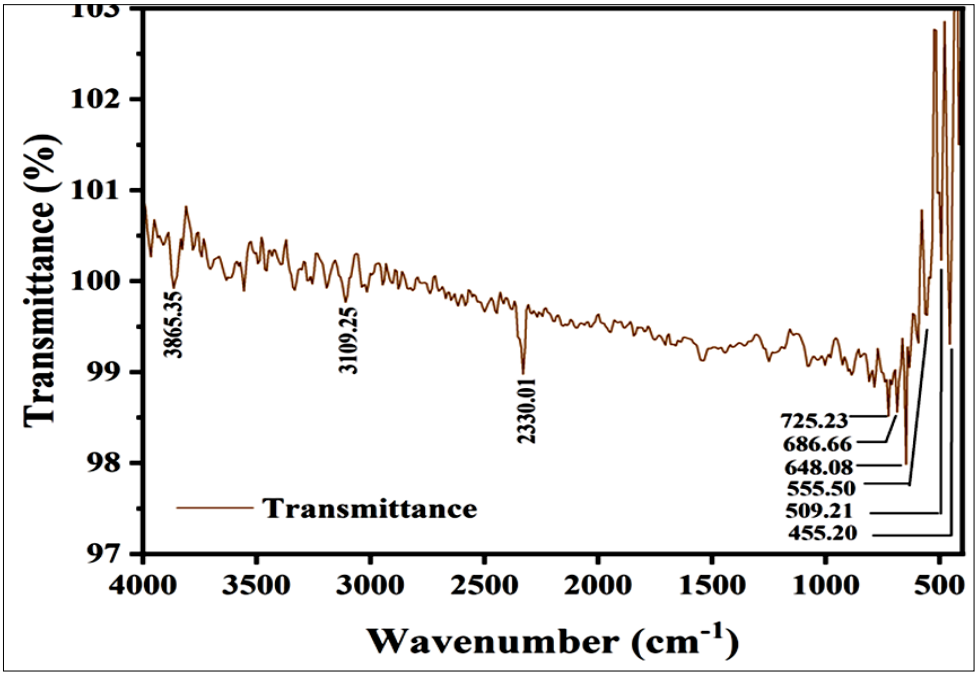

Fig. 2. FTIR spectra of sample 2 (chitosan-film with essential oil).

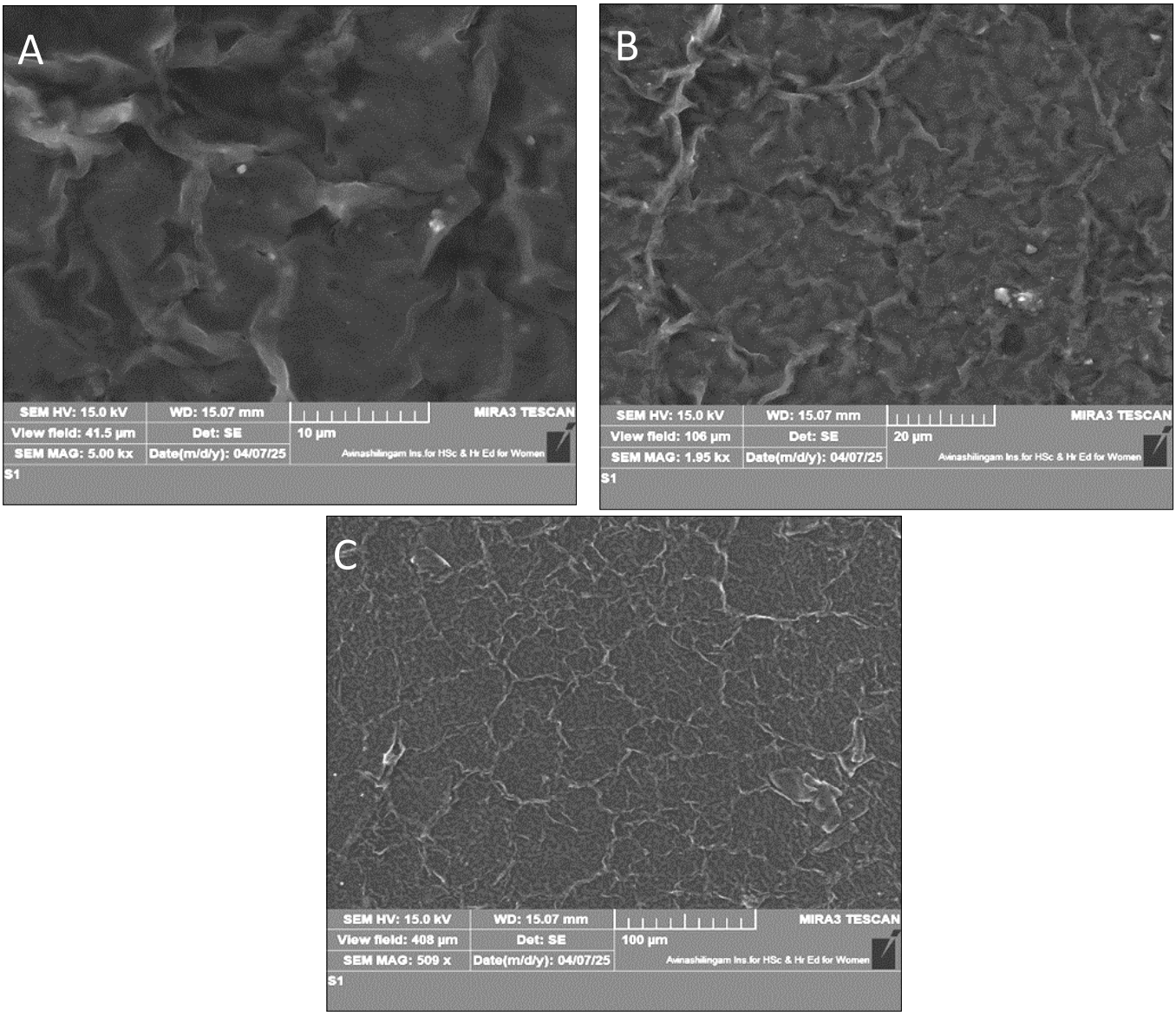

Fig. 3. SEM micrograph of sample 1 (Chitosan- film without essential oil) **A.** SEM image of the film surface at 5.00 kx magnification showing a relatively smooth and compact morphology with minimal cracks or pores; **B.** SEM image of the film surface at 1.95 kx magnification showing a slightly rough and uneven structure with minor surface irregularities; **C.** SEM image of the film surface at 509x magnification displaying an interconnected network-like structure with fine cracks across a larger area.

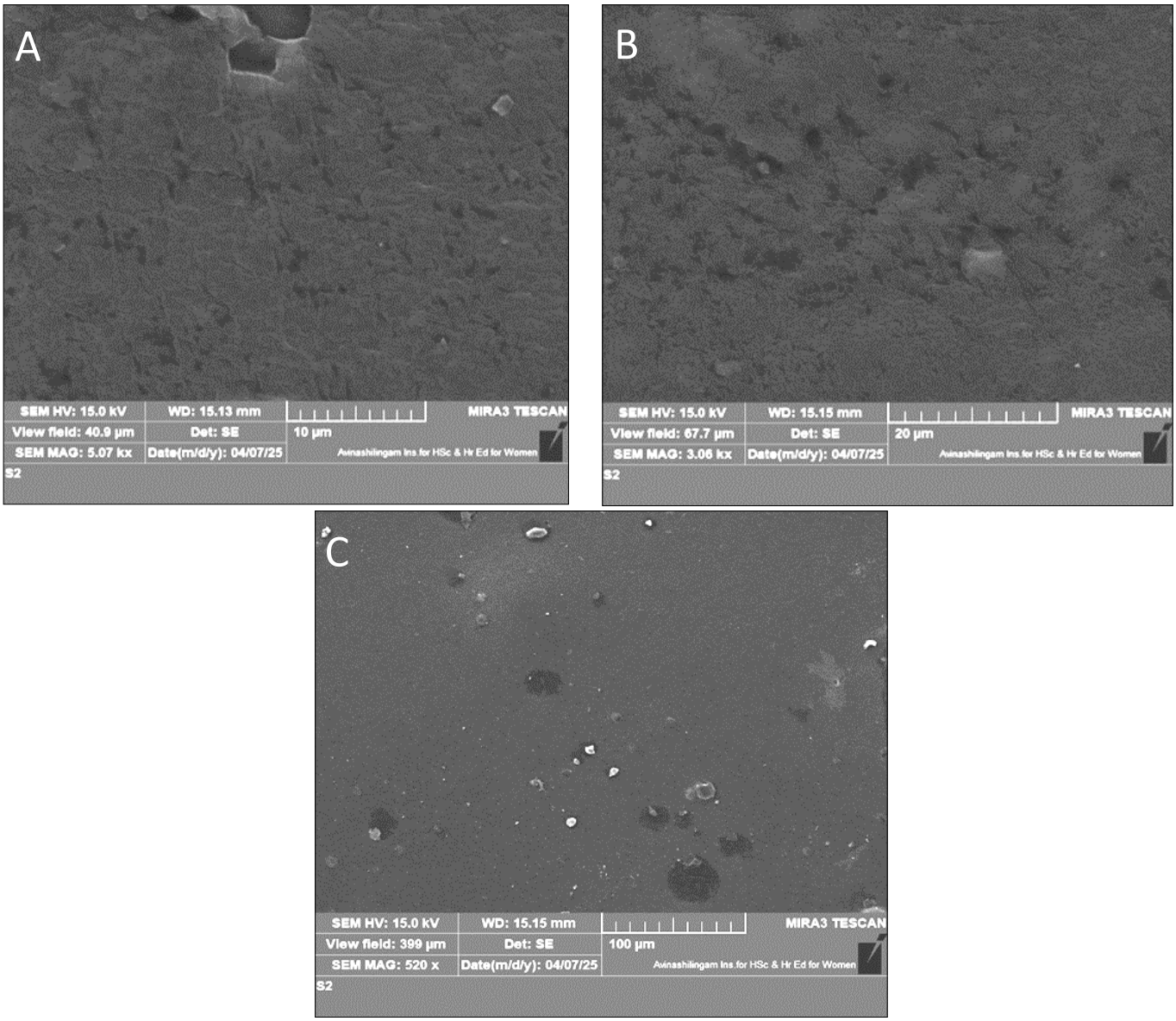

Fig. 4. SEM micrograph of chitosan- film with essential oil **A.** SEM image of the film sample at 5.07 kx magnification showing a relatively smooth surface with minor pits and dense packing of polymeric matrix; **B.** SEM image of the film surface at 3.06 kx magnification displaying a moderately rough texture with irregular surface morphology and embedded granules; **C.** SEM image at 520x magnification showing scattered particulate matter and surface.

Fig. 5. Antimicrobial activity of Sample1 Chitosan- film with essential oil-based solution against *Staphylococcus aureus* and *Escherichia coli*.

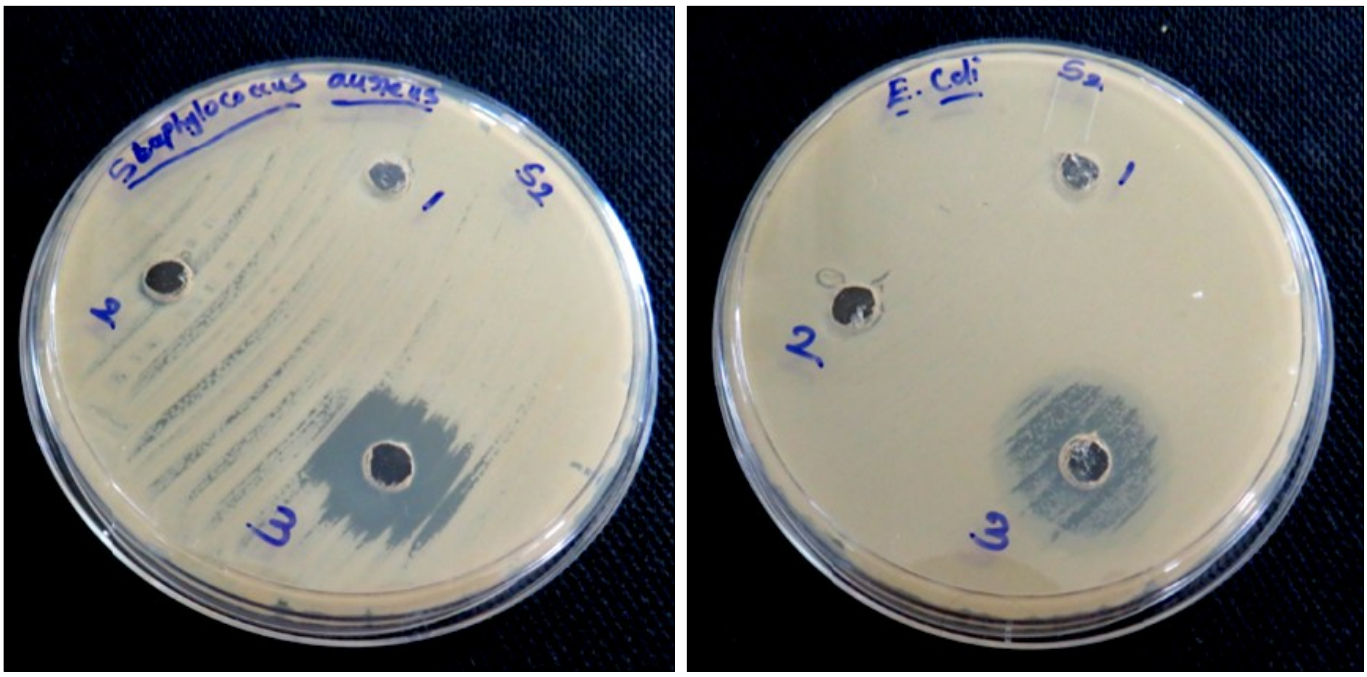

Fig. 6. Antimicrobial activity of sample 2 Chitosan- film with essential oil-based solution against *Staphylococcus aureus* and *Escherichia coli*.

Table 1. Antioxidant activity of standard ascorbic acid evaluated by DPPH assay

Concentration (µg/mL)	% Inhibition
3	62.30
6	70.49
9	81.97
12	90.16
15	95.90

Table 2. DPPH free radical scavenging activity of sample 2 at varying concentrations

Concentration (µL)	% Inhibition
10	76.22
50	74.59
150	71.31
250	68.03
350	64.75
500	63.11
750	56.55

Table 3. DPPH free radical scavenging activity of sample 1 at varying concentrations

Concentration (µL)	% Inhibition
10	75.40
50	74.59
150	70.49
250	69.67
350	68.85
500	59.83
750	52.45

Conclusion

The study highlights the potential of chitosan-based biopolymer film impregnated with essential oils as effective and sustainable alternatives to conventional plastic packaging. Functional groups and interactions between the chitosan, glycerol and essential oil components were successfully incorporated, as indicated by FTIR analysis. Meanwhile, SEM micrographs revealed a comparatively

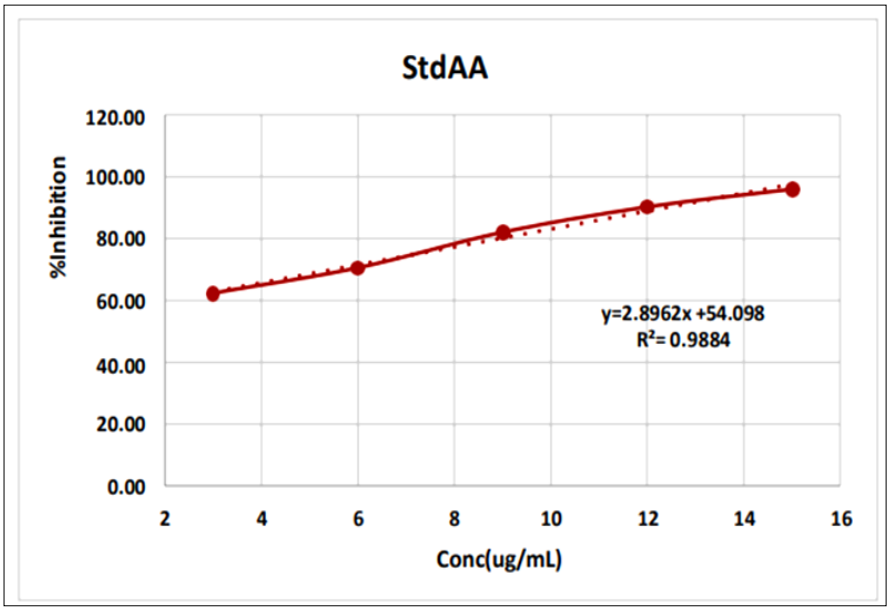

Fig. 7. Standard curve of ascorbic acid (AA) showing DPPH free radical scavenging activity at different concentrations.

smooth surface with slight variability, suggesting that the film's constituent parts were partially miscible. The films demonstrated considerable suppression against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E. coli*) bacteria at higher concentrations, according to the antimicrobial assays, particularly those loaded with essential oils (Sample 2). With sample 2 exhibiting marginally more antioxidant activity, both films also showed a noteworthy ability to scavenge free radicals. By offering antibacterial and antioxidant protection, our results demonstrate the potential of chitosan-essential oil films as environmentally responsible substitutes for increasing the shelf life of perishable goods.

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Authors' contributions

RT conceptualized the research article and designed the methodology for the literature search. RC guided the review work by formulating the review concept and approving the final manuscript. SP critically reviewed and edited the manuscript for intellectual content. AM helped in editing, summarizing and revising the manuscript. SM helped in editing, summarizing and revising the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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