Supporting Information

Neem Leaves Derived Carbon Dots Embedded Chitosan Based Active Films: A Sustainable Approach to Prolong the Shelf Life of Prawns

Ajitkumar Appayya Hunashyal[a], Saraswati P. Masti[a*], Lingaraj Kariyappa Kurabetta [b], Manjushree Nagaraj Gunaki[a], Suhasini Madihalli[a], Jennifer P. Pinto[b], Manjunath B. Megalamani[c], Bothe Thokchom[d], Ramesh Babu Yarajarla[d], Ravindra B. Chougale[e]

[a]Department of Chemistry, Karnatak Science College, Dharwad-580001, Karnataka, India

[b]Indian Institute of Packaging, Bengaluru, 562132, Karnataka, India

[c]Sustainable Energy & Nanomaterials Technology Research Applications Lab, Atria Institute of Technology, Bengaluru-560024, Affiliated to Visvesvaraya Technological University, Belagavi, Karnataka, India.

[d]Drosophila and Nanoscience Research Laboratory, Department of Applied Genetics, Karnatak University, Dharwad - 580003, India

[e]Department of Chemistry, Karnatak University, Dharwad-580003, Karnataka, India

Corresponding author e-mail ID: dr.saraswatimasti@yahoo.com

This file includes

S1. Compositional table

S2**.** Results of cytotoxicity studies on CPCD nanocomposite films

S3. TEM EDS, Histogram, and elemental mapping of NLCDs

S4. Characterisations of NLCDs

S5. Nanocomposite film characterizations

S6. Prawn packaging studies

S7. Statistical analysis

References

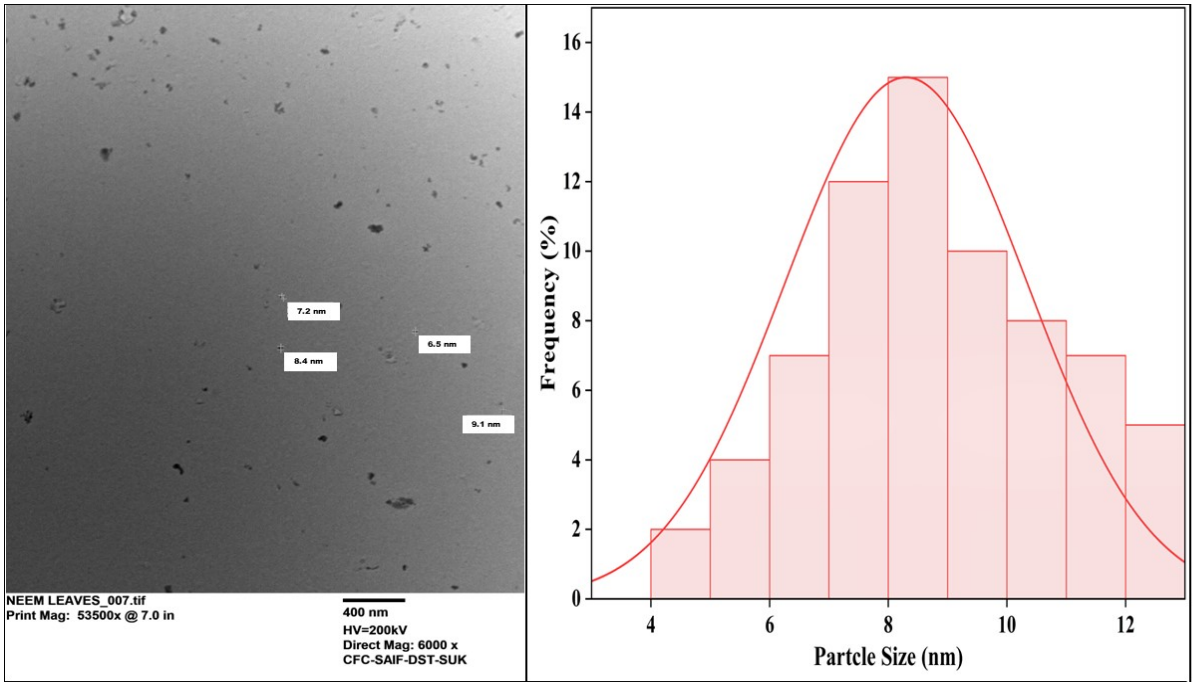
S1. Compositional Table

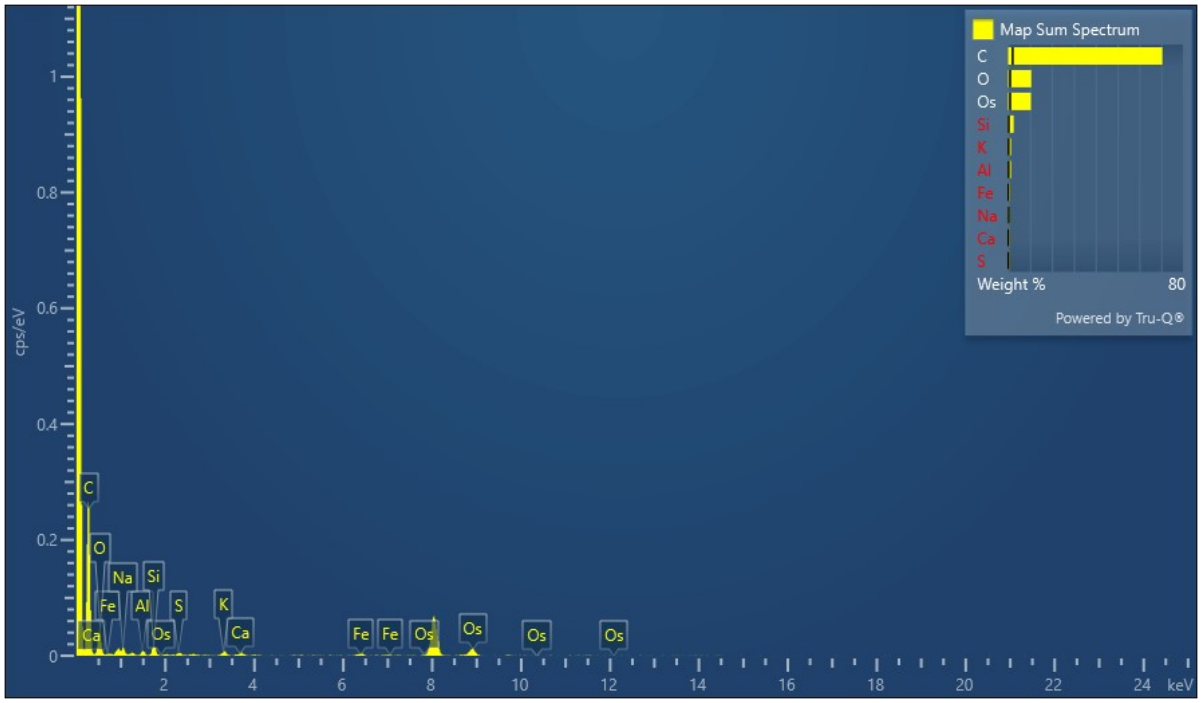
Sample code	CS (gm)	PVA (gm)	NLCD (mg)	Remarks
CP	1.5	0.5	-	Good film
CPCD-1	1.5	0.5	20	Good film
CPCD-2	1.5	0.5	60	Good film
CPCD-3	1.5	0.5	100	Good film

S2. Results of cytotoxicity studies on CPCD nanocomposite films

Sample	Cell Viability	Cytotoxicity Classification
Control	100 ± 1.2	
CP (neat film)	97 ± 1.8	Non-cytotoxic
CPCD-1	95 ± 1.7	Non-cytotoxic
CPCD-2	92 ± 2.5	Non-cytotoxic
CPCD-3	90 ± 2.1	Non-cytotoxic

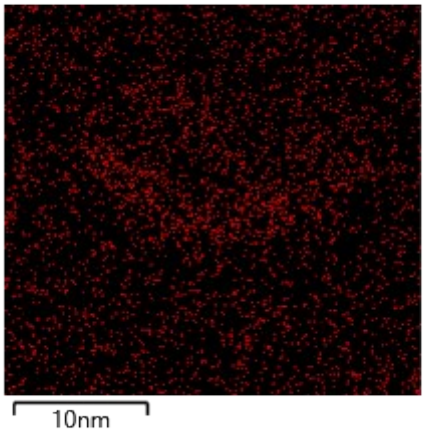
S3. TEM EDS, Histogram, and elemental mapping of NLCDs



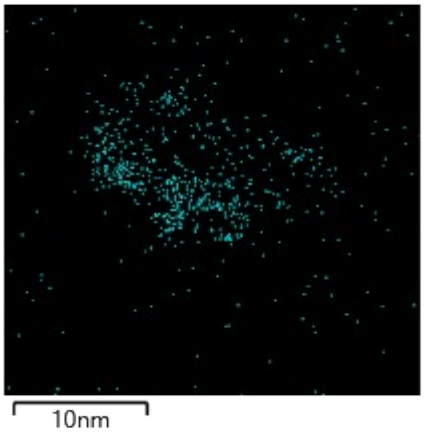


Map Sum Spectrum							
Element	Line Type	k Factor	k Factor type	Absorption Correction	Wt%	Wt% Sigma	Atomic %
C	K series	2.787	Theoretical	1.00	70.59	2.06	85.67
O	K series	2.033	Theoretical	1.00	10.58	0.96	9.64
Na	K series	1.203	Theoretical	1.00	0.90	0.31	0.57
Al	K series	1.052	Theoretical	1.00	1.28	0.22	0.69
Si	K series	1.000	Theoretical	1.00	2.52	0.30	1.31
S	K series	1.005	Theoretical	1.00	0.69	0.18	0.31
K	K series	1.012	Theoretical	1.00	1.29	0.21	0.48
Ca	K series	0.989	Theoretical	1.00	0.76	0.18	0.28
Fe	K series	1.131	Theoretical	1.00	0.94	0.20	0.24
Os	L series	2.172	Theoretical	1.00	10.44	0.89	0.80
Total:					100.00		100.00

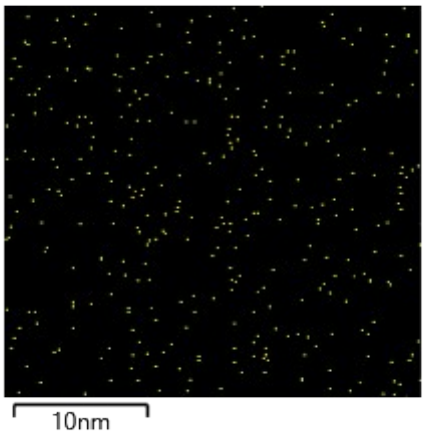
C Kα1_2



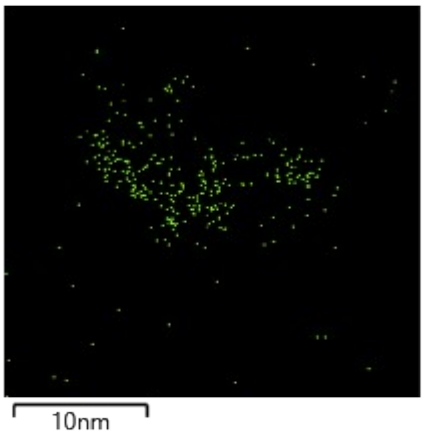
O Kα1



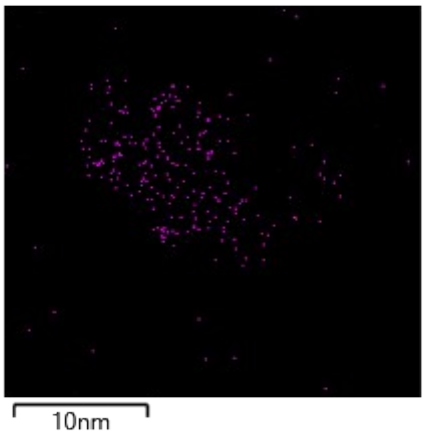
Os Lα1



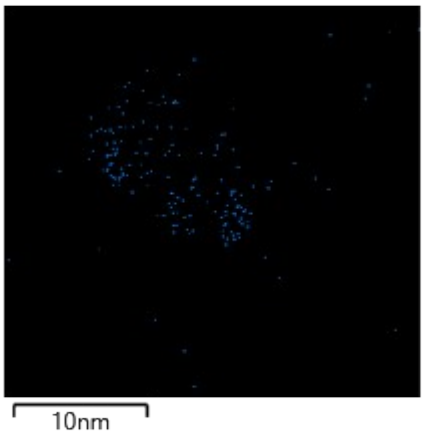
Si Kα1

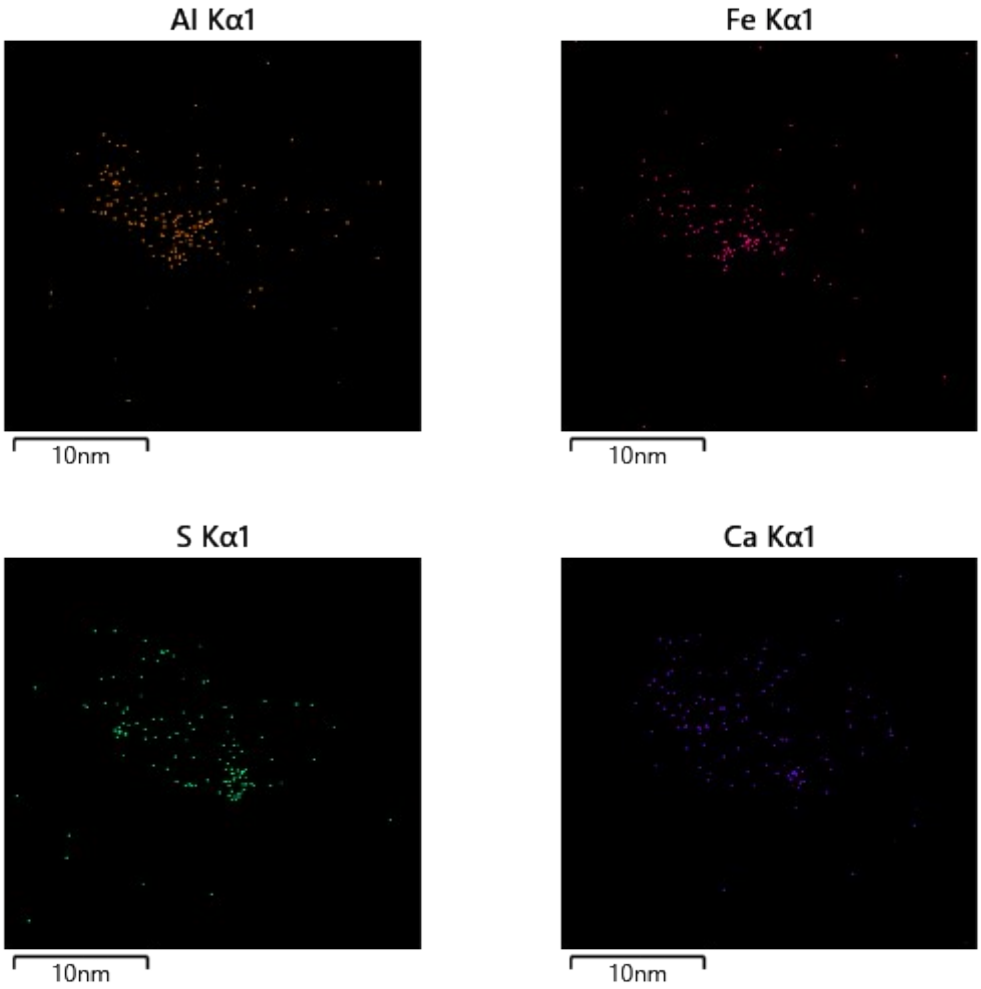


Na Kα1_2



K Kα1





S4. Characterisations of NLCDs

The absorption spectrum was checked using a UV-vis spectrophotometer (JASCO V-670 spectrophotometer, Japan). Fluorescence spectral measurements of aqueous NLCDs solution were monitored using a spectrophotometer (F-7000 FL Spectrophotometer). The morphology was examined by TEM-EDS and elemental mapping (Transmission Electron Microscope, JEOL JEM 2100 plus, Japan), and the particle size was evaluated using ImageJ. The chemical structures of the samples were analyzed using an attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectrophotometer (Thermo scientific Nicolet) in the range of 4000-500 cm^{-1} with an average resolution of 16 scans at 4 cm^{-1}. The interlayer spacing of the NLCDs was determined using X-ray diffractometry (XRD, Rigaku D/Max-IIA, Tokyo, Japan).

Using DPPH and H_2O_2 radical scavenging assays, the antioxidant characteristics of NLCDs at various concentrations (12.5, 25, 50, and 100 µg/mL) were evaluated. To prepare a solution for the DPPH assay, 4 mg of DPPH was dissolved in 100 mL of methanol. After that, 10 mL of the DPPH solution was diluted with 5 mL of various NLCD concentrations. For half an hour, the mixture was left at room temperature in the dark. The absorbance was measured using a

spectrophotometer at 517 nm. Using the following formula (1), the percentage of free radical scavenging activity was determined [1]:

$$\text{Free radical scavenging activity (\%)} = \frac{(A_c - A_s)x\ 100}{A_c} \quad (1)$$

A_c and A_s represent the absorbance of the neat solution (DPPH without NLCDs) and the test solution (DPPH with NLCDs), respectively. Phosphate buffer (pH 7.4) was used to prepare a 40 mM H_2O_2 solution for the H_2O_2 scavenging assay. A volume of 3.6 mL H_2O_2 solution was mixed with 2.4 mL of NLCD solutions at varying concentrations (12.5, 25, 50, and 100 µg/mL). For 10 min, the mixture was incubated at room temperature. The absorbance was determined using a spectrophotometer fixed at 230 nm. Equation (1) was used to calculate the H_2O_2 scavenging activity, where A_c was the plain solution's absorbance (H_2O_2 without NLCDs) and A_s was the test solution's absorbance (H_2O_2 with NLCDs)[23].

S5. Nanocomposite film Characterizations

Morphology, Structural and Optical Characterizations

Film specimens were vacuum sputter-coated with platinum for 180 seconds before testing. The film's microstructure was observed using SEM (JSM-IT500 operator JEOL) at an accelerating voltage of 1 kV. FTIR spectra of the film samples were recorded using an FTIR spectrometer (Thermo Scientific Nicolet) in attenuated total reflection (ATR) mode with the wavenumber ranging from 4000-500 cm^{-1} at 16 scan rates with the resolution of 4 cm^{-1}. A UV-vis spectrophotometer (JASCO V-670 spectrophotometer, Japan) was used to record the light transmittance spectrum of the film. The UV barrier and transparency properties of the film were also evaluated by measuring the percent light transmittance of the film sample (4 cm × 4 cm) at 280 nm (T_{280}) and 660 nm (T_{660}), respectively adopting previously reported methods [4]. The semi-crystalline nature of CS/PVA when combined with NLCDs was analyzed through X-ray diffraction (XRD) using a Rigaku D/Max-IIA instrument from Tokyo, Japan. Radiation was emitted from a Cu-Kβ source (λ = 1.5406 Å) at a high voltage of 40 kV and a current of 30 mA. The samples were scanned at a rate of 5° per minute across a 2θ range of 5° to 80°. The change in percentage crystallinity of the films were determined using previously reported methods [5].

S 5.1 Mechanical studies

The film sample was cut into rectangular strips (2 × 10 cm) using a high-precision double-blade cutter and the film thickness was measured using a digital micrometre (Mitutoyo Absolute, Japan) with an accuracy of 1 uM. The mechanical properties such as tensile strength (TS), elongation at

break (EB), and elastic modulus (EM) of the film were determined according to the standard method of ASTMD 882-88 using a Universal Testing Machine (UTM, DAK System Instrument, 7200 series, Mumbai). The machine was operated with an initial grip separation of 50 mm and a 50 mm/min crosshead speed.

S 5.2 Water vapour permeability (WVP) and water contact angle (WCA)

The assessment of water vapour permeation was performed according to a previously delineated protocol [6], albeit with certain modifications. Containers of amber hue with an average aperture diameter of 3.16 cm and a depth of 6.6 cm were opted for experimentation. These containers were loaded with anhydrous $CaCl_2$ to maintain 0% relative humidity (RH), with each container's mouth ensconced beneath a film specimen, leaving a 1 cm gap between the $CaCl_2$ and the film, securely tightened with Teflon tape. Subsequently, the containers were positioned within a desiccator filled with milli-Q water to uphold 100% RH. Alterations in container weight were meticulously monitored at 24-hour intervals over a span of 4 days, with slopes computed through linear regression ($R^2 \geq 0.982$). The water vapour transmission rate (WVTR) was quantified as the slope (g h^{-1}) divided by the surface area of the container aperture (m^2). Subsequently, water vapour permeability (WVP) was calculated employing the provided equation (2),

$$WVP = \frac{WVTR}{P}(RH_1 - RH_2).\chi \quad (2)$$

wherein P signifies the vapour pressure of water at 40°C (55.3 t), RH_1 represents the internal relative humidity within the container (0%), RH_2 denotes the external relative humidity outside the container (100%), and χ designates the thickness of the film specimen (meters). To investigate the surface wettability of the films, water contact angle measurements were performed using the sessile drop method using a contact angle meter Model DMs-401 (Kyowa Interface Science Co. Ltd., Tokyo), similar to our previous works[7]. Images were taken instantly after placing (2x2 cm) film samples on a sample holder and dousing them with double-distilled water. Four distinct places of the same film are averaged to get each contact angle value[5].

S 5.3 Release kinetic study of carbon-dot

Using food simulant solutions, the amount of NLCDs released from the films (CP, CPCD-1, CPCD-2, and CPCD-3) was measured[8]. To do this, a 100 mL conical flask filled with 20 mL of various food simulant solutions (10%, 50%, and 95% ethanol and water, respectively, to simulate alcoholic and aqueous food) was filled with the film sample (2 × 2 cm), which was then gently shaken and maintained at 25 °C. Using a UV-vis spectrophotometer, 1 mL of the solution was sampled at predefined intervals to measure the absorbance at 220 nm[9].

S 5.4 Antioxidant activity

DPPH and H_2O_2 radical scavenging assays were used to evaluate the films' antioxidant characteristics (CP, CPCD-1, CPCD-2, and CPCD-3). A fresh DPPH solution in methanol was made for the DPPH assay, and 20 mL of this solution was mixed with 100 mg of each film sample. After that, the prepared solutions were incubated for 6 h at room temperature. Using a spectrophotometer, the absorbance was determined at 517 nm after incubation[10]. A 40 mM H_2O_2 solution was made in phosphate buffer (pH 7.4) for the H_2O_2 scavenging assay. After being submerged in 10 mL of the H_2O_2 solution, each film sample, which weighed roughly 100 mg, was allowed to sit at room temperature for 1 h while being gently shaken. Using a spectrophotometer, the absorbance of these solutions was determined at 230 nm. The equation (3) was used to determine the percentage of free radical scavenging activity for each of the two assays[2]:

$$\text{Free radical scavenging activity (\%)} = \frac{(A_c - A_s)x\ 100}{A_c} \quad (3)$$

where A_S represents the test solution's absorbance (with films) and A_C represents the absorbance of the control solution (without films).

S 5.5 The antibacterial study

The antibacterial activity of different compositions of NCLDs against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) was evaluated using the agar well diffusion method, with few modifications as reported [11]. One colony of each test organism was cultured overnight in Luria-Bertani broth at 37°C on a shaker at 200 rpm. After incubation, a loopful of the bacterial culture was evenly spread onto nutrient agar plates. Wells (7 mm) were then prepared on the agar, into which 100 µL of CP, CPCD-1, CPCD-2, and CPCD-3 were added. The plates were incubated at 37°C for 24 hours, after which the zone of inhibition around each well was measured to assess the antibacterial activity.

S 5.6 Soil burial test

The degradability of the films we prepared was assessed using a soil burial test conducted in laboratory conditions, following the method outlined by Gunaki et al. [4]. Soil samples were obtained from the Botanical Garden on the Karnatak University Campus in Dharwad, Karnataka, India. We cut the samples into 2x2 cm squares and dried them at 40°C to determine their initial dry weight (w_1). These samples were then buried in the soil at a depth of approximately 8–10 cm below the surface. To maintain soil moisture, we sprayed water onto the soil surface. At an interval of 10

days, we removed the films from the soil, washed them, and dried them in an oven to obtain their final weight (w_2). Biodegradation was calculated using the formula (4),

$$\text{Biodegradation (\%)} = \frac{(W_1 - W_2)\, X\, 100}{W_1} \quad (4)$$

S6. Prawn packaging

Fresh prawns were procured from the local market and washed thoroughly under running tap water to remove surface debris, followed by rinsing with distilled water to ensure cleanliness. Excess moisture was carefully removed by blotting the prawns dry with sterile tissue paper. Sterile glass containers were prepared by autoclaving at 121°C for 15 minutes, followed by drying in a sterile environment to prevent contamination. The prepared prawns (5 g each) were allocated into four groups; each was assigned a different packaging condition. The first group was left uncovered, the second group was covered with commercial polythene, the third group was packaged with pristine CP films, and the fourth group was packaged with CPCD-3 films containing 5% NLCDs. All samples were stored at 4°C for 15 days to assess the effectiveness of different packaging materials in preserving the quality of the prawns. A total of five sets of each group were arranged, with each set designated to be opened only once for testing at specific intervals to maintain sample integrity.

S 6.1 P^H Studies

The p^H of the prawn samples was measured using 2 g of thoroughly mashed prawn, which was dissolved in deionised water using a mortar and pestle. The mashed solution was poured into a conical flask and diluted to a final volume of 100 mL, along with the rinse water used to clean the mortar and pestle. The mixture was allowed to settle before filtering, and a pH meter was used to determine the pH of the filtered solution[12].

S 6.2 TVB-N Studies

Targeting volatile amines like ammonia and other methylamines, the magnesium oxide method was used to calculate the Total Volatile Basic Nitrogen (TVB-N) level. Micro Kjeldahl equipment was used to conduct the examination. First, the prawn meat was mashed finely with a mortar and pestle. 10 g of that sample was added to a conical flask holding 100 mL of deionized water, following the Chinese standard protocol GB/T 5009.44-2003. The mixture was then heated for 30 minutes before being filtered. 10 mL of the filtrate was combined with 2-3 drops of phenolphthalein and put into a conical flask for analysis. 5 mL of that mixture and 5 mL of a suspended magnesium oxide solution were titrated against 0.01 mol/L hydrochloric acid. The following formula (5) was used to determine the TVB-N content:

$$TVB-N = \frac{(V_1 - V_2) \times C \times 14}{m \times 5/100} \times 100 \quad (5)$$

Where V_1 and V_2 are the volumes of hydrochloric acid used in the sample and control titrations, respectively, C is the hydrochloric acid concentration (mol/L), and m is the sample weight (g), and TVB-N is expressed as mgN/100 g[12].

S 6.3 TVC Studies

25 g of prawn meat were aseptically weighed and homogenized for one minute with 225 mL of sterile 0.1% physiological saline for the Total Viable Count (TVC) assay. For bacteriological analysis, the homogenized sample was thereafter serially diluted with 9 mL of sterile saline. The spread plate method on plate count agar was used to calculate the TVC[12].

S 6.4 Firmness and Odor Test

For the firmness test, prawns were removed from their packaging, and the texture was evaluated by applying uniform pressure to the dorsal region using gloved hands. The firmness was categorized, at a rating of 1 to 5 points, based on resistance to deformation, where higher resistance indicated firmer prawns. For the odor test, each sample was exposed to air for a brief period, and the odor was rated on a scale ranging from fresh, moderately off, to spoiled. Both tests were performed in a controlled environment to minimize external influences, with results recorded at the specified intervals. The tests were evaluated by a panel of trained assessors (n=5). The mode was taken as the final result.

S7. Statistical analysis

The replicated experimental units used for measuring the attributes of the films were measured for individually prepared films in triplicate. Using the SPSS statistical analysis software for Windows, a one-way analysis of variance (ANOVA) was carried out and each mean property value's significance ($p < 0.05$) was ascertained using Tuckey’s test.

References

1. Kurabetta LK, Masti SP, Gunaki MN, Hunashyal AA, Eelager MP, Chougale RB, et al. A synergistic influence of gallic acid/ZnO NPs to strengthen the multifunctional properties of methylcellulose: A conservative approach for tomato preservation. Int J Biol Macromol. 2024 Oct;277:134191.

2. Thokchom B, Bhavi SM, Abbigeri MB, Shettar AK, Yarajarla RB. Green synthesis, characterization and biomedical applications of Centella asiatica-derived carbon dots. Carbon Letters. 2023 Jun 6;33(4):1057–71.

3. Bhavi SM, Thokchom B, Abbigeri MB, Bhat SS, Singh SR, Joshi P, et al. Green synthesis, characterization, antidiabetic, antioxidant and antibacterial applications of silver nanoparticles from Syzygium caryophyllatum (L.) Alston leaves. Process Biochemistry. 2024 Oct;145:89–103.

4. Gunaki MN, Masti SP, D'souza OJ, Eelager MP, Kurabetta LK, Chougale RB, et al. Fabrication of CuO nanoparticles embedded novel chitosan/hydroxypropyl cellulose bio-nanocomposites for active packaging of jamun fruit. Food Hydrocoll. 2024 Jul;152:109937.

5. Pinto JP, Hiremani VD, D'souza OJ, Khanapure S, Narasagoudr SS, Goudar N, et al. Development of Chitosan-Copovidone nanocomposite films with antioxidant and antibacterial properties for food packaging applications. Food and Humanity. 2023 Dec;1:378–90.

6. Gasti T, Dixit S, Hiremani VD, Chougale RB, Masti SP, Vootla SK, et al. Chitosan/pullulan based films incorporated with clove essential oil loaded chitosan-ZnO hybrid nanoparticles for active food packaging. Carbohydr Polym. 2022 Feb;277:118866.

7. Pinto JP, D'souza OJ, Hiremani VD, Dalbanjan NP, Praveen Kumar SK, Narasagoudr SS, et al. Functional properties of taro starch reinforced polysaccharide based films for active packaging. Food Biosci. 2023 Dec;56:103340.

8. Roy S, Ezati P, Rhim JW. Gelatin/Carrageenan-Based Functional Films with Carbon Dots from Enoki Mushroom for Active Food Packaging Applications. ACS Appl Polym Mater. 2021 Dec 10;3(12):6437–45.

9. Khan A, Ezati P, Rhim JW. Chitosan/Starch-Based Active Packaging Film with N, P-Doped Carbon Dots for Meat Packaging. ACS Appl Bio Mater. 2023 Mar 20;6(3):1294–305.

10. Kurabetta LK, Masti SP, Eelager MP, Gunaki MN, Madihalli S, Hunashyal AA, et al. Physicochemical and antioxidant properties of tannic acid crosslinked cationic starch/chitosan based active films for ladyfinger packaging application. Int J Biol Macromol. 2023 Dec;253:127552.

11. Abbigeri MB, Thokchom B, Bhavi SM, Singh SR, Joshi P, Yarajarla RB. Potential in vitro antibacterial and anticancer properties of biosynthesized multifunctional silver nanoparticles using Martynia annua L. leaf extract. Nano-Structures & Nano-Objects. 2024 Sep;39:101320.

12. Wei L, Yuanyuan H, Yanping C, Jiaojiao J, Guohua H. *Penaeus orientolis* prawn freshness rapid determination method based on electronic nose and non-linear stochastic resonance technique. Bioengineered. 2015 Jan 2;6(1):42–52.