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RESEARCH ARTICLE
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Development of Antibacterial Biodegradable Film using Polyvinyl Alcohol, Carboxymethyl Cellulose and *Annona muricata* Leaves Extract Composite Incorporated with Chitosan

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ABSTRACT

This study aims to develop and characterize antibacterial biodegradable film using polyvinyl alcohol (PVOH), carboxymethyl cellulose (CMC), and *Annona muricata* leaf extract (15%) reinforced with chitosan (0, 1, 2, and 4%). Surface morphology and biomaterial interaction were accessed using Scanning Electron Microscope (SEM) and Fourier Transform Infrared Spectroscopy (FTIR), respectively. Film reinforced with 4% chitosan showed the greatest thickness ($P<0.05$) compared to others. The elongation at break (EAB) of *Annona*-based film reinforced with 1% chitosan was considerably higher ($P<0.05$) than controls. The *annona*-based film reinforced with 4% chitosan demonstrated significantly higher opacity ($P<0.05$) than controls. The film was completely degradable in the soil on day 16. *Anonna*-based film inhibited *R. planticola, C. farmeri, C. braakii, A. hydrophila* and *S. lentus.* Furthermore, an increased chitosan concentration showed increased antibacterial activity of the film. Therefore, *Annona*-based film reinforced with 4% chitosan shows promising potential as a biodegradable packaging material.

Keywords: *Annona muricata*, Chitosan, Biofilm, Antimicrobial, Biodegradable.

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INTRODUCTION

Global plastic production has reached approximately 320 million tons per year, driven by increasing demand (Asgher et al., 2020). Single-use plastics, often used for packaging, are typically discarded after use, with a minimal percentage being recycled. Plastics take several years to decompose, causing environmental hazards (Jeevahan and Chandrasekaran, 2019). Research by Hermawan et al. (2019); Jafarzadeh et al. (2021) highlights the environmental issues associated with synthetic packaging materials, primarily due to their non-biodegradability. This has spurred research and innovation in biodegradable packaging, which offers a more sustainable alternative. Biodegradable materials decompose quickly and reduce environmental pollution (Dilkes-Hoffman et al., 2018; Sani et al., 2021). Biodegradable packaging is derived from three main components; proteins, polysaccharides, and lipids (Sani et al., 2021). Besides covering and protecting the food from external contaminants, packaging has evolved to prolong the shelf-life and slow food deterioration (Bhargava et al., 2020; Maciel et al., 2020). In this regard, natural antioxidants and antimicrobial compounds have been incorporated into the packaging solution to prepare the active film for packaging (Koirala et al., 2025a). *Annona muricata* has been reported to have 212 bioactive compounds (Coria-Téllez et al., 2018). According to Ibrahim et al. (2022) *A. muricata* leaves contain antioxidant compounds including flavonoids, glycosides, terpenoids and steroids. Gyesi et al. (2019)

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added that *Annona* essential oil has high antioxidant capabilities of 50.88g AAE $100g^{-1}$. Furthermore, studies by González et al. (2017) stated that the total phenolic compound (TPC) for *Annona* leaves was recorded at 3.24–3.95g 100 g^{-1} dried weight. Antioxidants may reduce the oxidation of fats and oils, minimize the deterioration process, extend the shelf life, increase the stability of fats, and prevent loss of sensory and nutritional quality (Hasmila et al., 2019).

On the other hand, chitosan is well known for its biocompatibility, physicochemical, mechanical properties, and various industrial applications (Koirala et al., 2025b; Nirmal et al., 2024). Chitosan is a deacetylated product of chitin which can be obtained from crustaceans and mollusk byproducts (Koirala et al., 2024). Chitosan can be used in various food industry applications including antimicrobial agents, biodegradable food packaging material, fat replacers, carriers of active compounds, etc. (Koirala et al., 2024). Kumari and Kishor (2020) demonstrated that chitosan effectively inhibits the growth of spoilage bacteria, including *Salmonella* sp. and *Lactobacillus fructivorans*. Chitosan film, when used as a coating, can enhance the sensory qualities of food and provide antibacterial benefits, which improve both the visual appeal and health attributes of the product for consumers (Abdallah et al., 2017). Nevertheless, the chitosan-based film faces challenges, including brittleness, low resistance and poor thermal stability (Koirala et al., 2024; Koirala et al., 2025a). Hence, various strategies have been employed to enhance the efficacy of chitosan including nanoengineering, active site modification, the addition of other active compounds, etc (Koirala et al., 2023; Koirala et al., 2024). For example, reinforcing chitosan-based films with glycerol, polyvinyl alcohol and carboxymethyl has been reported to enhance their mechanical properties (Giannakas et al., 2016; Cazon et al., 2020; Nguyen et al., 2022). Therefore, the study aims to develop *Annona*-based film reinforced with chitosan and evaluate micromorphology, physicochemical, mechanical, and antibacterial properties. Additionally, biodegradation tests of the prepared films have been conducted for environmental safety.

MATERIALS & METHODS

Materials

Methanol (CH_3OH, purity: 99.9%, MW=32.04), glycerol (purity: 99.5%, MW=92.09), sodium hypochlorite ($NACIO_2$, MW=74.44), plate count agar, nutrient agar, Mueller-Hinton (MH) agar and 0.5 McFarland standard, were purchased from EMD Milipore (Merck), Germany. Oxytetracycline antibiotic discs (OT 30µg) were purchased from Liofilchem, Italy.

Polyvinyl alcohol (PVOH, full hydrolyzed) $4.23x10^6$ kDa calculated by using the Mark–Houwink–Sakurada equation i.e., $[\eta] = K \times M^a$. Where K, a, [η], and M are the constant for solvent, polymer shape factor, intrinsic viscosity, and molecular weight of PVOH, respectively. Carboxymethyl cellulose (CMC, Viscosity: 2%, H_2O, 25°C, MW= $8.29x10^4$ kDA calculated by using the Mark–Houwink–Sakurada equation i.e., $[\eta] = K \times M^a$. Where K, a, [η], and M are the constant for solvent, polymer shape factor, intrinsic viscosity and molecular weight of CMC, respectively.

The degree of substitution (DS) was determined by using the acid-wash method and was calculated using the Equation:

$$\text{Degree of substitution (DS)} = \frac{162 \times \%CMC}{[5900 - (58 \times \%CMC)]} \quad (1)$$

where carboxymethyl content, (% CMC) was calculated using Equation (2)

$$\%\ CMC = [(V_0 - V_n)\ M \times 0.059 \times 100] \quad (2)$$

Where V_0 is the amount of hydrochloric acid used to titrate the blank solution, V_n is the amount of hydrochloric acid used to titrate samples, M is the molar concentration of hydrochloric acid used, and m is the sample amount. The value of 162 $g{\cdot}mol^{-1}$ is the molar mass of the anhydroglucopyranose unit (AGU), and 58 $g{\cdot}mol^{-1}$ is the molar mass of $–CH_2COOH$.

Therefore, DS of CMC was 1.12

Sample Preparation of Annona Muricata Extract

Annona leaf extraction was prepared according to Ibrahim et al. (2022). Approximately 30±0.5g of *A. muricata* powder was mixed with 200mL methanol-distilled water (6:4). The mixture was shaken with Stuart Orbital Shaker (Fisher Scientific, Malaysia) for 24h followed by filtration using Whatman filter paper number 1. The excessive solvent was removed by drying the extract using a rotary evaporator R-210 (Buchi, Switzerland). The extract was stored in a vial at 4ºC prior experiment.

Shrimp Shell Preparation and Chitosan Extraction

Shrimp shells were collected from a shrimp processing plant in Johor, Malaysia. Shrimp shells were washed under running water and subsequently with a 2.5% sodium hypochlorite solution. The materials were pulverized using an industrial blender and sieved through a 16-mesh knit (Santos et al., 2019). Shrimp shell powder was subjected to deproteinized, demineralization, deproteinization and deacetylation to obtain the chitosan. Chitosan solution was prepared according to Kaya et al. (2018b).

Chitosan (MW= $1.82x10^5$ kDa) was calculated by using the Mark–Houwink–Sakurada equation i.e., $[\eta] = K \times M^a$. Where K, a, [η], and M are the constant for solvent, polymer shape factor, intrinsic viscosity, and molecular weight of chitosan, respectively. The degree of deacetylation of chitosan (DA) was 81.11±0.64 %.

Film Preparation

The film was prepared by mixing 2±0.1g of polyvinyl alcohol (PVOH), and 1±0.1g of carboxymethyl cellulose (CMC) at 70°C for 45 minutes. After that, *Annona* extracts at 15% concentration and 1% (w/v) glycerol were added to the mixture. Additionally, chitosan (0, 1, 2, and 4 %) was added to the solution. The mixture (10mL) was formed on a petri dish before drying (Memmert UF110, Germany) at 30°C with 10% fan speed for 48 hours.

Scanning Electron Microscope (SEM)

Film strips (1cm×1cm) were cryofracture by immersion into liquid nitrogen and conditioned in a

desiccator at 25°C before measurement. Conditioned samples were mounted on the specimen holder using double-sided adhesive tapes before sputter coated with gold under vacuum. *Annona*-based film samples were characterized by a field emission scanning electron microscope (SEM, S-4800, Czech Republic) at an accelerating voltage of 15kV and observed using magnifications 500 and 1500 (Liu et al., 2019).

Fourier Transform Infrared Spectroscopy (FTIR)

An aliquot of sample was positioned in the infrared spectrophotometer, utilizing the single bounce attenuated total reflection (ATR) method (Ibrahim et al., 2022). The Origin Pro version 2016 software was employed for data collection and analysis. The functional groups present in the film constituents were isolated based on their peak ratio.

Film Thickness and Moisture Content

Film thickness was determined by using digimatic micrometer (Mitutoyo APB-3D, Japan) (Hazirah et al., 2016). Moisture content was determined following the method by Sharma et al. (2021). Small pieces of samples (2×2 cm) were weighted (W_1) before being dried at 105°C (Binder ED, Germany). After heating, the samples were weighted (W_2) and moisture content (%) was calculated as follows:

$$Moisture\ content\ (\%) = \frac{W_1 - W_2}{W_1} \times 100$$

W_I: Initial weight of samples
W_2: Final weight of samples

Tensile Strength (TS) and Elongation at Break (EAB)

Mechanical properties were determined according to ASTM (2013) method. Small pieces of samples (1×8cm) were placed at 60mm clamp distance, 1mm s^{-1} stretching speed and 10mm length of the texture analyzer (TA XT Plus, Stable Micro Systems, Surrey, UK). TS and EAB were calculated as follows:

$$Tensile\ strength\ (MPa) = \frac{P}{b \times d}$$

P is maximum load (N); *b* is sample width (mm); and *d* is film thickness (mm).

$$Elongation\ at\ break\ (\%) = \frac{L - L_0}{L_0} \times 100$$

L_0 is the initial length of films; L is the length of films at breaks

Color and Opacity

The color parameters were determined by using a colorimeter (Chromameter CR-400, Japan). The total color difference (ΔE) was calculated as follows:

$$\Delta E^2 = [(\Delta L *)^2 + (\Delta a *)^2 + (\Delta b *)^2]^{0.5}$$

Small pieces of samples (1x6cm) were placed into a cuvette and analyzed for light transmission in the range of 200-800nm using a UV–visible spectrophotometer (UV-1800, Shimadzu, Japan). The opacity of samples was calculated at a transmittance of 600nm as follows:

$$O = \frac{Abs600}{d}$$

O is the opacity; A_{bs600} is the absorbance value at 600nm; d is film thickness (mm).

Water Solubility of the film

Films were prepared by drying to constant weight in the oven (Binder ED, Germany) at 50°C. Then, the films were cut into 2×3cm strips and put in a petri dish with 30mL of deionized water before incubating for 48 hours. The films then were retrieved and dried at 50°C for another 24 hours (De Carli et al., 2022). The films were weighed and the weight loss was calculated based on the following equation:

$$Water\ solubility\ (\%) = \frac{W_1 - W_2}{W_1} \times 100$$

W_I: Initial weight of samples
W_2: Final weight of samples

Biodegradation Test

Film strips were prepared as stated in 3.3.10. The dried films then were buried at 5cm below the soil surface in plastic containers filled with 10cm thickness of organic soil before being incubated at 25°C for 20 days. 10mL of water was added to each container daily to keep the soil moisture at 40%. Every four days, the films were retrieved from the soil, cleaned and weighed. The films weight loss then was calculated using the following formula:

$$Weight\ loss\ (\%) = \frac{W_1 - W_2}{W_1} \times 100$$

W_I: Initial weight of samples
W_2: Final weight of samples

Antimicrobial Test

The agar disk diffusion method was employed to conduct the antimicrobial test. The single colony bacteria were then mixed with 1mL of distilled water in a vial to ensure that the concentration of bacteria equated to 0.5 McFarland standard. This concentration was measured using a densitometer (Biomerieux, France). Approximately 0.1mL of the solution was pipetted onto Mueller Hinton (MH) agar before spread using sterilized L-shaped glass. A film strip measuring 2cmx2cm, along with an oxytetracycline antibiotic disk as the positive control was placed onto the agar surface before incubated for 24 hours. Following incubation, the inhibition zone surrounding the antibiotic was measured.

Statistical Analysis

One-way ANOVA was conducted using SPSS software (Statistical Package for the Social Sciences) to analyze the variance. Means were compared using Tukey's Test, with a significance level set at $P<0.05$. All analyses were performed in quintuplicate.

RESULTS AND DISCUSSION

Structural Properties

Scanning Electron Microscope (SEM)

Annona-based film reinforced with chitosan exhibited a smooth and dense morphology, with no visible holes or cracks (Fig. 1). In controls (0% chitosan), the particles are evenly distributed throughout the film. As the concentration of chitosan increased, an increasing number of small white particles became apparent. This coagulation is likely due to the relatively weak interaction between

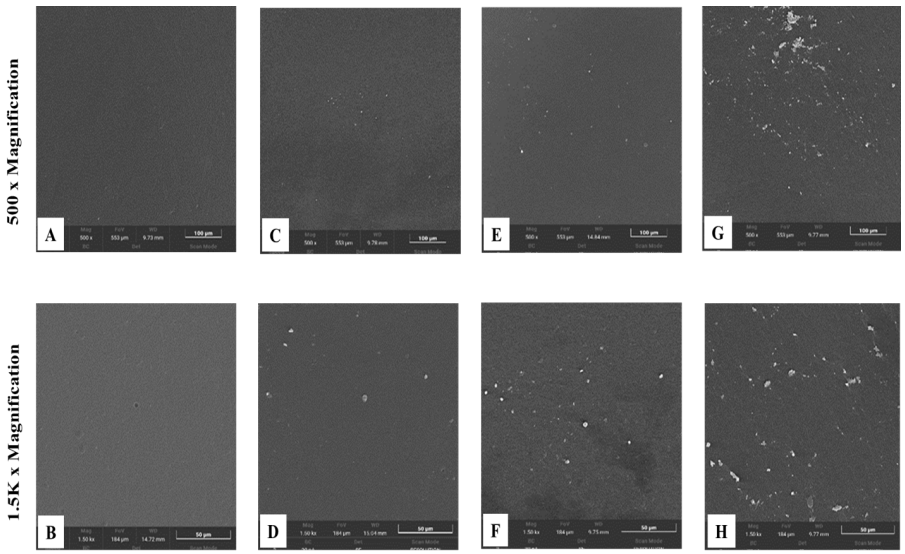

Fig. 1: SEM images of *Annona*-based film surfaces (A & B) *Annona*-based film without chitosan, (C & D) *Annona*-based film with 1% chitosan, (E & F) *Annona*-based film with 2% chitosan, and (G & H) *Annona*-based film with 4% chitosan.

Annona and chitosan (Wen et al., 2023). Numerous studies (Jakubowska et al., 2021; Zhang et al., 2022) stated that the white spot is attributed to a significant number of insoluble chitosan particles that are not fully dispersed within the film matrix. Comparable surface morphologies were observed in other studies using SEM to investigate the incorporation of plant extracts into chitosan blend films (Kaya et al., 2018a; 2018b).

Fourier Transform Infrared Spectroscopy (FTIR)

Seven functional groups were identified in *A.muricata* based film aromatic group (663.51cm^{-1}), ester group (1041.56cm^{-1}), ether group (1257.59cm^{-1}), alkane group (1373.32cm^{-1}), alkene group (1597.06cm^{-1}), carboxylic acid group (2939.52cm^{-1}), and hydroxyl group (3286.70cm^{-1}) (Fig. 2). This is consistent with the findings of Daud et al. (2016) and Ibrahim et al. (2022) where five and seven functional groups were obtained from *A. muricata* leaves extract, respectively. The initial spectrum showed a broad band at 3286.70cm^{-1} within the range of 3600-3100cm^{-1}, indicating the presence of N-H and hydrogen-bonded hydroxyl groups (Sun et al., 2017; Ren et al., 2017). The peak at 1519.91cm^{-1} in films with chitosan shifted from two to one upon removal of chitosan, indicating the loss of the N-H bond associated with the amide group in chitosan (Fig. 2).

Physicochemical Properties

Thickness and Moisture Content

Annona-based film without chitosan exhibited significantly lower thickness (P<0.05) compared to *Annona*-based film reinforced with 1, 2 and 4% chitosan (Table 1). The thickness of the film increased with increasing chitosan concentration (P<0.05). Notably, *Annona*-based film reinforced with 4% chitosan demonstrated the highest thickness value (P<0.05), surpassing the others. The chitosan reinforcement significantly affects the physical dimensions of the *Annona*-based film. In addition, *Annona*-based film can be categorized as a thin film (≤0.25mm) standardized by the ASTM (1985). According to Hazirah et al. (2016), thickness depends on the total weight and interaction of the film compositions (Hu et al., 2022). Studies by Bonilla et al. (2013); Valenzuela et al. (2013) documented that thickness are increasing with increasing chitosan ratio and thus produce more compact film network. Therefore, *Annona*-based film reinforced with 4% chitosan produced the greatest crosslinking in the matrix and thereby contributed to the most compact film network formation.

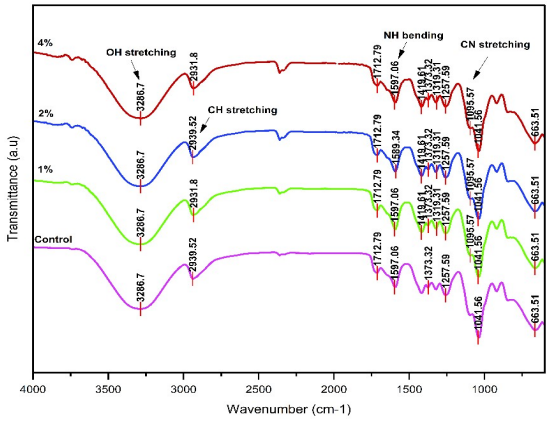

Fig. 2: FTIR spectra for *Annona*-based film samples. Control (*Annona*-based film with 0% chitosan), 1% (*Annona*-based film with 1% chitosan), 2% (*Annona*-based film with 2% chitosan) and 4% (*Annona*-based film with 4% chitosan).

Table 1: Thickness, moisture content, tensile strength and elongation at break of film samples at different chitosan concentrations

Film chitosan (%)	Thickness (mm)	Moisture content (%)	Tensile strength (Mpa)	Elongation break (%)
0	0.063±0.000^{d}	21.5±3.53^{b}	0.041±0.0075 ^{a}	250.3±6.10^{b}
1	0.071±0.001^{c}	27.5±1.40^{a}	0.029±0.0062 ^{a}	438.5±17.60^{a}
2	0.076±0.001^{b}	24.7±0.76^{ab}	0.028±0.0010 ^{a}	368.8±21.26^{ab}
4	0.080±0.001^{a}	24.9±1.68^{ab}	0.028±0.0057 ^{a}	355.5±22.24^{ab}

Results as expressed means±standard deviation. Mean values in the same column with different superscript letters ($^{a-d}$) are significantly different (P<0.05).

Annona-based film exhibited a noticeably lower moisture content (P<0.05) compared to *Annona*-based film reinforced with 1% chitosan (Table 1). However, the moisture content of *Annona*-based film reinforced with 1%

chitosan was not significantly different (P>0.05) compared to *Annona*-based film reinforced with 2 and 4% chitosan. This indicates that increasing the concentration of chitosan beyond 1% does not significantly affect the moisture content of the film. Moisture content refers to the volume of water molecules within the film's structure (Xu et al., 2021). In this study, *Annona*-based films exhibited slightly higher moisture contents compared to chitosan-based films, which had a moisture content ranging from 15-20% (Leceta et al., 2013). In addition, Nguyen et al. (2020) reported that 1% chitosan-based film had a moisture content of only 18%. Various researchers documented chitosan films with moisture content exceeding 20% typically contain glycerol, suggesting that these films are plasticized (Pereda et al., 2009; Hirase et al., 2010; Aguirre-Loredo et al., 2016).

Color and Opacity

The L*, which measures the lightness, was significantly lower (P<0.05) in *Annona*-based film compared to those reinforced with 1, 2, and 4% chitosan (Table 2). Interestingly, *Annona*-based film without chitosan exhibited the lowest green hue (-0.6±0.05^{a}) (P<0.05) compared to the chitosan-reinforced films. However, the a* value, which indicates redness or greenness, showed no significant difference (P>0.05) between *Annona*-based film reinforced with 1, 2 and 4% chitosan. In contrast, the b* value, which represents yellowness or blueness, was significantly higher (P<0.05) in the control compared to *Annona*-based film reinforced with chitosan. Furthermore, the total color change (ΔE*) was significantly higher (P<0.05) in the controls compared to *Annona*-based film reinforced with chitosan. This suggests that the addition of chitosan affected the lightness and color properties of the film, particularly influencing the green and yellow hues.

Table 2: Color and opacity of the film samples with different chitosan concentrations

Film	Color				Opacity
chitosan (%)	*L**	*a**	*b**	*ΔE**	
0	79.2±0.58^{b}	-0.6±0.05^{a}	32.7±0.32^{a}	31.0±0.46^{a}	4.32±0.08^{b}
1	88.3±0.41^{a}	-1.05±0.03^{c}	4.05±0.12^{b}	1.10±0.20^{b}	4.77±0.83^{ab}
2	88.7±0.49^{a}	-0.96±0.04^{bc}	3.92±0.19^{b}	2.02±1.84^{b}	4.94±0.79^{ab}
4	88.8±0.19^{a}	-0.87±0.07^{b}	4.10±0.19^{b}	1.89±1.43^{b}	6.87±1.35^{a}

Results expressed as means ± standard deviation. Mean values in the same column with different superscript letters ($^{a-c}$) are significantly different (P<0.05).

The low L* values observed in *Annona*-based film without chitosan indicate a darkening effect likely attributed to the presence of *Annona* leaf extract and drying temperature effects (Srikandace, 2019). The a* values across all samples leaned towards greenness, consistent with the natural green color of the *Annona* leaves extract. Additionally, the *Annona*-based film without chitosan displayed the highest b* value, which can be attributed to the presence of flavonoids known for imparting a yellow hue to the materials (Cruz et al., 2020; Salim et al., 2022). Therefore, the color characteristics of the films are likely influenced by the botanical properties of the *Annona muricata* leaf extract and the flavonoid present, emphasizing the impact of natural components on the film's coloration.

The opacity of the films increased gradually with higher chitosan concentration, but there were no significant differences (P>0.05) between the control films and those reinforced with 1 and 2% chitosan. However, the *Annona*-based film reinforced with 4% chitosan exhibited significantly higher opacity (P<0.05) compared to the controls, indicating that 4% chitosan notably enhanced the film's opacity. This enhancement is likely due to the greater presence of compounds within the film. Kaya et al. (2018a) documented that increased opacity in chitosan films is associated with the presence of pigments and tints on the film surface. According to Srikandace (2019), while transparent film may appeal to customer preference, opaque films are crucial as light barriers. Excessive light transmission through the film can promote oxidation reactions, leading to product deterioration (Bajić et al., 2019; Salari et al., 2021; Costa et al., 2021; Salim et al., 2022). Therefore, films with higher UV-blocking capabilities are preferred for packaging, as they offer better protection against oxidation and extend the product's shelf life (Bajić et al., 2019; Salim et al., 2022). Opacity is crucial in packaging materials, impacting their ability to protect contents from light and preserve product integrity.

Water Solubility of Films

The water solubility rate was recorded at 43.1, 31.8, 23.0, and 20.5% for *Annona*-based films reinforced with 0, 1, 2, and 4% chitosan, respectively (Fig. 3). The annona-based film reinforced with 0% chitosan showed the highest rate of solubility. This could be due to the presence of phenolic compounds in *A. muricata* which have hydroxyl groups in their structure. The hydroxyl groups are known to increase the solubility of the films (Mir et al., 2018). Meanwhile, *Annona*-based film reinforced with 4% chitosan showed significantly lower (P<0.05) solubility compared to film of 0% chitosan. This value was in range with the value of chitosan-based film enriched with propolis extract at approximately 22% (De Carli et al., 2022), chitosan film with mango leaf extract at 27.4% (Rambabu et al., 2019) and chitosan film with 4% *Ficus carica* Linn leaves extract at 20.3%. Kaya et al. (2018b) stated that in general, chitosan film was hydrophobic and incorporation with fruit extract led to a lower solubility level of the film. This happened due to the bonding between the extract and the chitosan chain, which decreased their attraction to water molecules (Kaya et al., 2018b).

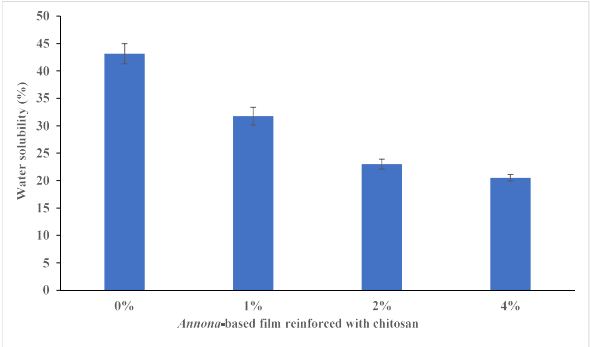

Fig. 3: Water solubility of *Annona*-based film reinforced with chitosan.

Mechanical Properties

Tensile Strength (TS) and Elongation at Break (EAB)

Tensile strength (TS) showed no significant difference (P>0.05) among all samples (Table 1). TS value decreased with increasing chitosan concentration, attributed to the formation of intra-molecular hydrogen bonds (Ren et al., 2017). Additionally, elongation at break (EAB) for *Annona*-based films (controls) was significantly lower (P>0.05) compared to *Annona*-based films reinforced with 1% chitosan (Table 1). However, *Annona*-based films reinforced with 1% chitosan showed no significant difference (P>0.05) to *Annona*-based films reinforced with 2 and 4% chitosan. EAB of *Annona*-based films reinforced with chitosan was notably improved due to chitosan's presence and its interaction facilitates chain sliding, enhancing the film's overall flexibility and chain mobility. Nguyen et al. (2022) added that glycerol serves as an effective plasticizer, contributing to the film's flexibility and durability. Glycerol reduces intermolecular interaction, softens rigidity and decreases brittleness (Chillo et al., 2008; Liu et al., 2013; Nguyen et al., 2022). Overall, *Annona*-based film demonstrates superior mechanical properties compared to other food packaging that has a tensile strength of 5–15MPa and an EAB at approximately 200% (Zhang et al., 2020).

Biodegradation Test

The soil biodegradation test showed that all *Annona*-based films reinforced with chitosan degraded at day 20 (Fig. 4). At day 16, *Annona*-based film reinforced with 4% chitosan exhibited significantly the highest (P<0.05) biodegradation rate with 100% compared to other films. This finding aligns with a study conducted by Kaya et al. (2018a), which reported that the biodegradation rate of 98.8% for chitosan-leaf film is within 15 days. A study done by Gasti et al. (2020) stated that the presence of natural bioactive compounds in leaf extract like polyphenol somehow boosts soil microflora activity which speeds up biodegradation, and soil microbes using chitosan and *Solanum nigrum* L leaf extract as carbon and nitrogen sources for growth. This finding also echoed some previous works done by Riaz et al. (2020) using chitosan-based film with *Ficus carica* Linn leaves extract and Yilmaz et al. (2022) using chitosan-based film with Chinese chive root extract. Besides, the water diffusion into the chitosan polymer matrix can cause swelling, which increases the film's biodegradation (De Carli et al., 2022).

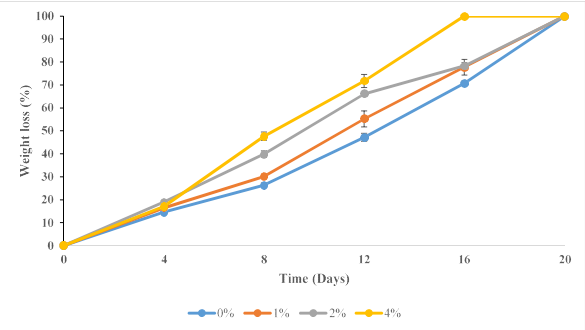

Fig. 4: Biodegradation rate of *Annona*-based film reinforced with chitosan after 20 days.

Antimicrobial Activity

Annona-based films were susceptible to all tested five bacteria; *R. planticola, C. farmeri*, *C. braakii*, *A. hydrophila* and *S. lentus* (Table 3). The inhibition zone of *Annona*-based film reinforced with 4% chitosan was significantly (P<0.05) more susceptible against *R. planticola, C. farmeri, S. lentus* compared to others. However, the inhibition zone for *C. braakii* and *A. hydrophila* showed no significant (P>0.05) difference in all treatments. Nevertheless, *Annona*-based film had an antimicrobial activity against all bacteria tested. *Annona*-based films without chitosan (0%) showed the smallest inhibition diameter (17.7±1.5mm) against Gram-positive *Staphylococcus lentus*, compared to others that are mostly Gram-negative bacteria. This echoed previous studies where the inhibition zone for a Gram-positive bacterium, *Staphylococcus aureus* is comparatively smaller than that of a Gram-negative bacterium, *E. coli* (Chang et al., 2021; Ahmad & Sarbon et al., 2021; Zhang et al., 2022; Eelager et al., 2023; Lee et al., 2023). This is due to the presence of thicker peptidoglycan layers of Gram-positive bacteria in their cell walls (Siripatrawan & Kaewklin, 2018; Hezma et al., 2019) which functions as a barrier to reduce the penetration of the films. Consequently, antimicrobial treatments tend to be less effective against Gram-positive bacteria, resulting in smaller zones of inhibition.

Table 3: Antibacterial activity of *Annona*-based film reinforced with 0, 1, 2 and 4% chitosan against five different bacteria and oxytetracycline (OT) antibiotic. The inhibition zone (mm) of the oxytetracycline range is as follows: (R≤14, I = 15-18, S≥19)

Bacteria	Diameter of inhibition zone (mm)				
	OT	0%	1%	2%	4%
Raoultella planticola	28	21.0±1.7^{b}	23.3±0.6^{b}	27.0±2.6^{ab}	34.7±5.7^{a}
Citrobacter farmeri	20	21.0±1.0^{b}	24.0±3.5^{b}	27.3±2.9^{b}	42.7±2.5^{a}
Citrobacter braakii	23	24.0±1.7^{a}	29.6±1.5^{a}	30.3±4.9^{a}	31.7±3.1^{a}
Aeromonas hydrophila	22	23.3±0.6^{a}	24.0±2.0^{a}	24.3±2.9^{a}	26.3±2.5^{a}
Staphylococcus lentus	30	17.7±1.5^{b}	20.0±2.0^{b}	24.0±2.7^{ab}	30.3±4.0^{a}

Results are expressed as means ± standard deviation. Mean values in the same row with different superscript letters ($^{a-b}$) are significantly different (P<0.05).

Generally, the inhibition zone increases with increasing percentage of chitosan. This shows that chitosan benefits as antimicrobial activity. There were several mechanisms reported for the antimicrobial activity of chitosan (Inanli et al., 2020). The electrostatic interactions between the positively charged chitosan groups (the amine groups of glucosamine) and the negatively charged groups on microbial cell membranes disrupt the membrane integrity, leading to increased permeability and ultimately causing cell death. Changes in the permeability of the cell wall due to this interaction cause proteinaceous materials and other intracellular components to leak (Hosseinnejad & Jafar, 2016). The second process is the interplay between the DNA of bacteria and the hydrolysis products that permeate into the microbial environment. Because of this interaction, mRNA synthesis is inhibited, which in turn causes disruptions in the synthesis of proteins and impairs the activity of different enzymes. Essentially, microbes' ability to manufacture vital proteins and enzymes required for their survival and growth is impaired by the hydrolysis

products derived from chitosan, which interfere with their genetic machinery.

Conclusion

The *Annona*-based film reinforced with 4% chitosan represents an optimal formulation, effectively balancing the film's strength and flexibility of the film. *Annona*-based film reinforced with 4% chitosan is thicker and exhibits a slightly higher tensile strength, making it more resilient and capable of withstanding mechanical stress before breaking. The EAB in *Annona*-based film reinforced with 4% chitosan suggests it is sufficiently flexible to handle without tearing. Additionally, its increased opacity reduces light transmission, helping to prevent lipid degradation. Both Annona extract and chitosan contribute positively to the film's physical and mechanical properties. *Anonna*-based film inhibited *R. planticola, C. farmeri, C. braakii, A. hydrophila* and *S. lentus*. Consequently, the *Annona*-based film reinforced with 4% chitosan shows great potential as a nature-based primary packaging material.

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