

RESEARCH ARTICLE

Mechanistic insights into antibacterial and antioxidant bacterial cellulose films functionalized with *Vitex negundo* extract

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Abstract - As the life sciences embrace sustainability, biocompatible and biodegradable materials with minimal ecological footprints are emerging as key enablers in various applications. Addressing the growing demand for sustainable and functional biomaterials, this study investigated how the incorporation of *Vitex negundo* (VN) extract influences the structural, chemical, and functional characteristics of alginate–bacterial cellulose (AB) films especially its antibacterial and antioxidant potential. AB films were prepared with varying concentrations of VN extract (0.25% and 0.5%). Physical characterization was performed through thickness measurement and Fourier-transform infrared spectroscopy (FTIR). The antibacterial potential was evaluated through the inhibition zone method, while the antioxidant potential was assessed using the DPPH radical scavenging assay. The findings contribute to the design of plant-extract-functionalized biopolymer films, underscoring the importance of extract concentration optimization. The results support the potential of VN–BC films as bioactive materials for various applications, while highlighting directions for future studies on mechanical stability, release kinetics, and industrial scalability.

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1. Introduction

The transition from petroleum-based to bio-based materials represents a pivotal development in sustainable innovation. These bio-based materials are primarily derived from renewable polymers, notably biopolymers such as polysaccharides and proteins [1]. Compared to plant-derived polysaccharides, bacterial cellulose (BC) offers distinct advantages in purity, sustainability, and production efficiency. Produced extracellularly by genera such as *Gluconacetobacter* and *Komagataeibacter*, BC consists solely of β -(1 \rightarrow 4)-linked D-glucose chains and is free of hemicellulose and lignin. Its structural homogeneity, robust mechanical properties, and shorter fermentation cycles enable cost-effective large-scale manufacturing [2,3]. Despite its favourable physical and structural attributes, BC lacks intrinsic antimicrobial, antioxidant, or anti-inflammatory properties [4]. Conversely, BC exhibits a crystalline structure and adaptable surface chemistry, enabling the development of multifunctional composites by integrating bioactive agents [5]. Functionalization strategies encompass in situ modification, which is governed by production parameters, and ex situ modification, which is performed after bacterial cellulose synthesis. Both types of modifications yield tailored functionalities and expand the material's application potential [6]. This has spurred growing interest in embedding natural plant extracts into BC matrices to synergistically combine mechanical robustness with biological activity.

Rich in phytochemicals with antimicrobial, antioxidant, and anti-inflammatory properties, plant extracts present a sustainable and biocompatible means of enhancing bacterial cellulose functionality. BC-based composites incorporating bioactive compounds have been widely explored, and plant-derived extracts are increasingly recognised as a viable and effective option [7]. Existing literature has provided valuable insights into specific aspects. Incorporation of phytochemicals from green tea leaves, roselle flower petals, and *Hibiscus rosa-sinensis* L. flowers has been shown to impart both antioxidant and antibacterial properties to BC matrices [8]. Similarly, BC membranes infused with bioactive constituents from sage and neem demonstrated pronounced antibacterial effects, highlighting their potential for biomedical and food-related applications [7]. Further studies have reported that BC enriched with extracts from tulsi, brahmi, lemon, blackberry, nettle root, and nettle leaves, as well as with essential oils, exhibited inhibitory activity against *Cronobacter* species and other reference strains [3]. Among the wide spectrum of botanicals explored, *Vitex negundo* (VN) has not been incorporated into bacterial cellulose films. *V. negundo*, commonly known as Nirgundi, is a medicinal plant with a wide range of therapeutic applications, especially for its strong antioxidant and antibacterial properties. It has been used in combination with sodium alginate to create biopolymer films for biomedical applications that demonstrate good antimicrobial properties, particularly under dry conditions [9]. While VN has not been specifically incorporated into bacterial cellulose films, its successful integration into other biopolymer films and strong antimicrobial properties suggest it could be a viable additive for BC films in future research. Thus, this study aimed to provide insights into the BC films functionalized with VN extract by evaluating their antibacterial and antioxidant activities, while employing FTIR analysis to characterise the functional groups responsible for these enhanced properties.

2. Materials and Methods

2.1 Sample Collection and Preparation

V. negundo leaves were collected from a residential area in Kuantan, Pahang. The freshly collected samples were thoroughly washed under running water to remove surface dirt and debris. To eliminate residual moisture, the plant material was dried in a hot-air oven at 60 °C until a constant weight was achieved. The dried material was subsequently ground into a fine powder using a laboratory blender. The resulting powdered *V. negundo* leaves were stored in a sealed container prior to subsequent analyses.

2.2 Microbial culture

The bacterial cellulose (BC)-producing strain *Komagataeibacter nataicola* was inoculated in yeast–glucose–calcium carbonate (YGC) medium. The medium composition consisted of 50 g/L glucose, 12.5 g/L calcium carbonate (CaCO₃), and 5 g/L yeast extract. The pH of the prepared medium was adjusted to 5.0 using 2 M acetic acid prior to sterilisation and inoculation [10].

2.3 BC production, Harvesting, and Purification

An overnight culture of *K. nataicola* was used as the inoculum for bacterial cellulose (BC) production. A 10 mL aliquot of the culture was transferred into freshly prepared YGC medium and incubated under aerobic conditions in conical flasks. The cultures were maintained statically at 25 °C for seven days, during which BC pellicles formed at the air–liquid interface. The harvested pellicles were purified by washing under running tap water, followed by soaking in 0.1 M sodium hydroxide at 80 °C for 2 h. The samples were subsequently rinsed with deionised water until a neutral pH was attained. Finally, the purified pellicles were given a final rinse and stored in a chiller to prevent contamination and to preserve moisture content [11].

2.4 Soxhlet Extraction of *Vitex negundo* leaves

Methanolic extraction of plant material was carried out using a Soxhlet apparatus. A total of 200 g of ground plant powder was placed into a thimble, and 50 mL of methanol was added as the extraction solvent. The extraction was continued until the solvent in the round-bottom flask turned dark green, indicating saturation with plant constituents. The resulting extract was collected, and its volume was measured prior to concentration. The solvent was removed using a rotary evaporator until a viscous, honey-like residue remained. The crude methanolic extract was subsequently transferred into glass vials and stored at 4 °C until further use.

2.5 Preparation of Alginate–Bacterial Cellulose–*Vitex negundo* Extract (ABV) Films

An alginate solution was prepared by dissolving 2 g of sodium alginate in 160 mL of 3% (v/v) glacial acetic acid and stirring continuously for 8 h at room temperature (25 °C). Separately, a bacterial cellulose (BC) solution was obtained by dispersing 10 g of BC in 40 mL of 3% (v/v) glacial acetic acid. The BC solution was then added to the alginate solution under continuous stirring for 2 h at room temperature, followed by thorough mixing. Glycerine, at 20 wt.% of the combined dry weight of alginate and BC, was incorporated as a plasticiser and stirred for an additional 1 h [12]. *V. negundo* extract was subsequently introduced into the mixture to achieve final concentrations of 0.25% and 0.5% (w/w), relative to the dry weight of alginate and BC. The composite solution was stirred for another 8 h to ensure homogeneity. A 60 mL volume of the prepared mixture was then cast into sterile Petri dishes with a 10 cm radius and dried at 45 °C for 15 h. The resulting films were carefully peeled off and stored under refrigerated conditions until further analysis.

2.6 Physical Characterisation

The thickness of the ABV films was measured using digital callipers with an accuracy of 0.01 mm. The moisture content of the film was measured using the direct drying method. Film samples of 30 mm × 30 mm were initially weighed (M_0) and then dried in an oven at 105 °C until a constant weight was achieved. All measurements were conducted in triplicate to ensure accuracy. The moisture content of the film was calculated using Eq. (1):

$$MC(\%) = \frac{m_0 - m}{m_0} \times 100\% \quad (1)$$

where m_0 represents the initial mass of the film samples, while m denotes the mass of the samples after drying.

The molecular vibrations and polar functional groups of the films were analysed using a Fourier transform infrared (FTIR) spectrometer (Nicolet Instrument, Thermo Co., Madison, WI, USA). Film specimens measuring 2 cm × 2 cm were scanned in the spectral range of 4000–700 cm⁻¹ at a resolution of 4 cm⁻¹. The thermal stability of the films was measured using a thermogravimetric analyser at temperatures between 30 °C to 1000 °C, with a heating rate of 10 °C per minute and a nitrogen flow rate of 20 mL per minute.

2.7 Antimicrobial Activity

The ABV films were evaluated for antimicrobial activity against the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus subtilis*. Both bacterial strains were activated using the shaking flask culture method at 37 °C for 24 h. The bacterial suspensions were standardised to 0.5 McFarland turbidity (approximately 3 × 10⁸ CFU/mL) by diluting with normal saline and adjusting the absorbance to 0.8–1.3 at 625 nm.

The standardised bacterial inoculum was evenly spread onto the surface of nutrient agar plates. Film samples (6 mm diameter) were sterilised under ultraviolet light for 30 min and carefully placed onto the inoculated agar surfaces. The plates were incubated at 37 °C for 12 h, after which the antimicrobial activity was determined by measuring the diameter of the inhibition zones formed around the films. Each measurement was repeated at least 3 times to ensure accuracy [12]. The inhibition zone width (W) was calculated using Eq. (2):

$$W = \frac{(D - d)}{2} \quad (2)$$

where W represents the width of the inhibition zone, D is the outer diameter of the inhibition zone, and d is the inner diameter of the film sample.

2.8 Antioxidant Activity

The antioxidant activity of the films was determined according to the method described by [13]. Briefly, 0.5 g of each film sample was mixed with 5 mL of deionised water and incubated under shaking conditions for 24 h to obtain the test solution. After centrifugation, the resulting supernatants were collected and used for the free radical scavenging assay. A 20 μ L aliquot of the supernatant was added to 5 mL of DPPH solution prepared in 95% ethanol. The reaction mixture was incubated in the dark at 25 °C for 30 min, and the absorbance was measured at 517 nm. The DPPH radical scavenging rate was calculated using equation (3):

$$\text{DPPH radical scavenging rate}(\%) = \frac{(A_0 - A)}{A_0} \times 100\% \quad (3)$$

where A_0 represents the absorbance of the control, and A represents the absorbance of the film sample.

It is recommended that the authors provide adequate information to enable the work to be replicated. Methods that have previously been published should be referenced, and only relevant modifications should be mentioned.

3. Results and Discussion

3.1 Thickness and Moisture Content of the Casted Film

The thickness of ABV films exhibited a pronounced, non-linear dependence on the concentration of the VN extract, as shown in Table 1. The unmodified control film (AB) recorded a thickness of 0.19 mm. When VN was incorporated at 0.25%, the thickness increased to 0.28mm, and with 0.5% VN, it reached a maximum of 0.32mm. These gains relative to the control suggest that a low-to-moderate addition of VN enhances the structural buildup of the biopolymeric network.

The decrease in moisture content enhances the film's barrier properties, making it more suitable for applications requiring low water permeability. The results show a clear trend of reduced moisture content with increasing concentrations of VN extract. The control film exhibits high moisture content, reflecting its hydrophilic nature due to the composition of its base materials. Incorporating 0.25% VN extract results in a significant reduction in moisture content, by approximately 9.83%. This demonstrates that VN effectively lowers the film's moisture retention. Increasing the concentration to 0.5% further reduces the moisture content by an additional 3.24%. This highlights a concentration-dependent effect. VN extract contains bioactive compounds, such as flavonoids and essential oils, which likely impart hydrophobic properties to the film [14]. These compounds reduce the film matrix's affinity for water molecules, thereby decreasing moisture content. In addition to this effect, VN may also enhance the film's functionality by introducing antimicrobial and antioxidant properties. These features, combined with reduced moisture content, improve the film's potential for applications such as food packaging. In summary, the incorporation of VN significantly reduces the film's moisture content in a concentration-dependent manner, acting as an effective hydrophobic modifier. These results suggest the film is well-suited for applications that require reduced moisture content and improved barrier properties

Table 1. Thickness of films prepared with different VN concentrations

Sample	Thickness (mm)	Moisture content (%)
Control (AB)	0.25 \pm (0.02)	98.88 \pm (0.97)
0.25 % VN	0.28 \pm (0.02)	81.43 \pm (0.61)
0.5% VN	0.32 \pm (0.01)	78.75 \pm (2.85)

3.2 Antimicrobial Activity

The diameter of the inhibition zone on *B. subtilis* plates after incubation with ABV films (Figure 1) increased from 0.1 mm to 0.37 mm (Table 2), equivalent to 23.33% increase as the VN extract increased to 0.5 %. Similarly, the diameter of the inhibition zone on *E. coli* plates after incubation with ABV films (Figure 2) increased from 0.1 mm to 0.28 mm (Table 3), representing a 12% increase as the VN extract concentration increased to 0.5%. The increase in inhibition zone diameters for both Gram-negative and Gram-positive bacteria with higher VN extract concentrations in ABV films indicates improved antimicrobial activity against these bacteria. The bioactive components in VN extract, such as flavonoids and phenolic compounds, might be responsible for this increase. In line with previous studies, VN reportedly has strong antibacterial activity against both Gram-negative and Gram-positive bacteria, including *E. coli* and *B. subtilis* [15].

The antibacterial activity of ABV films against both Gram-positive and Gram-negative bacteria increases with higher VN extract concentration, with a stronger effect against the Gram-positive bacterium *B. subtilis*. This suggests that these

films could be used to develop antimicrobials. Gram-positive bacteria may be more sensitive to the antimicrobial chemicals in the VN extract, as evidenced by the greater increase in the inhibition zone for *B. subtilis* compared with *E. coli*. The structural differences between the cell walls of Gram-positive and Gram-negative bacteria may account for this differential vulnerability. The outer membrane of Gram-negative bacteria can act as a barrier to certain antimicrobial agents, thereby reducing their effectiveness [16].

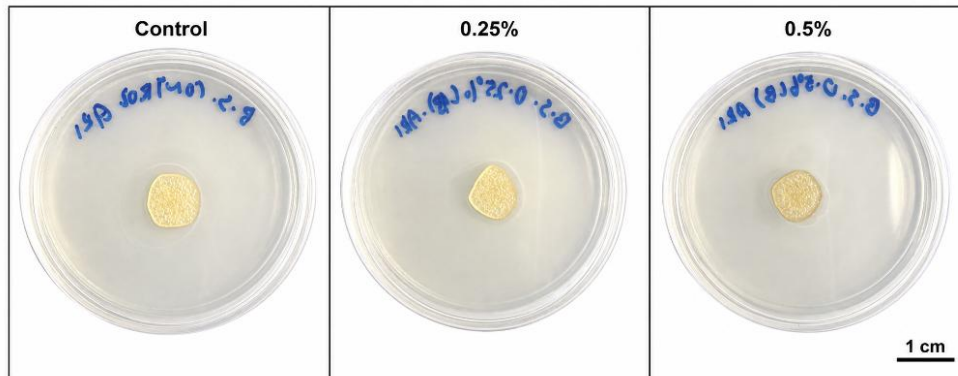


Figure 1. The inhibitory zone diameters of *B. subtilis* of the ABV films from left to right are control, centre is 0.25%, and right is 0.5%

Table 2. The width of the inhibitory zone of *B. subtilis* of the films

Sample	W (mm)
Control (AB)	0.1 ± (0.01)
0.25 % VN	0.30 ± (0.02)
0.5% VN	0.37 ± (0.02)

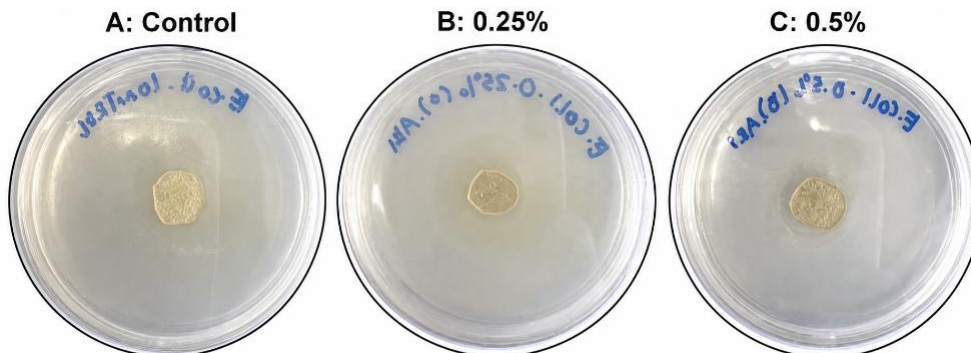


Figure 2. The inhibitory zone diameters of *E. coli* of the ABV films from left to right are control, centre is 0.25%, and right is 0.5%

Table 3. The width of the inhibitory zone of *E. coli* in the films

Sample	W (mm)
Control (AB)	0.1 ± (0.02)
0.25 % VN	0.25 ± (0.00)
0.5% VN	0.28 ± (0.03)

3.3 Antioxidant activity

The incorporation of *V. negundo* extract into bacterial cellulose–alginate (BC) films markedly enhanced their antioxidant potential, as measured by DPPH radical scavenging activity (Table 4). VN contributes antioxidant phenolics that boost radical scavenging. This functional contribution is consistent with the FTIR spectra. While the control film showed no detectable activity, VN-loaded samples exhibited concentration-dependent responses. At 0.25% VN, films displayed moderate scavenging (54.9%), which further increased at 0.5% VN (72.9%).

Table 4. DPPH radical scavenging activity of bacterial cellulose–alginate films with varying concentrations (0 – 0.5%)

Sample	DPPH radical scavenging rate (%)
Control (AB)	N/A
0.25 % VN	54.94 ± (1.01)
0.5% VN	72.92 ± (1.55)

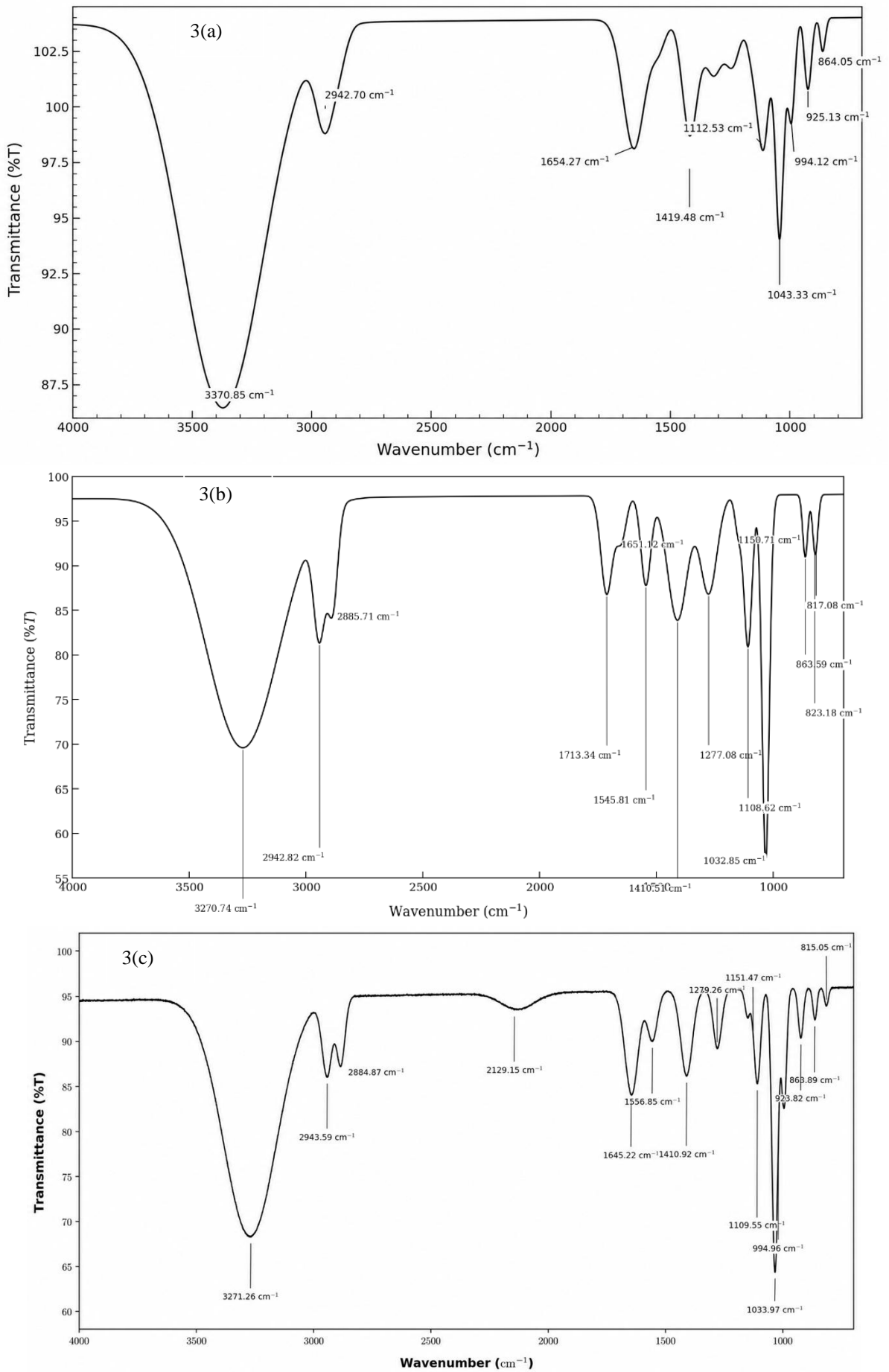


Figure 3. FTIR spectra of bacterial cellulose–alginate films containing different concentrations of VN extract (0 – 0.5%); 3(a) Control; 3(b) 0.25 % VN extract; 3(c) 0.5% VN extract

3.4 Fourier-Transform Infrared Spectroscopy

FTIR spectra of ABV films (Figure 3) showed the characteristic absorption bands of polysaccharides, including a broad O–H stretching vibration near 3270–3370 cm^{-1} , C–H stretching around 2885–2943 cm^{-1} , an amide I region at 1645–1655 cm^{-1} and intense C–O–C vibrations in the 900–1150 cm^{-1} region. These bands were consistently present across all films (summarised in Table 5), confirming the preservation of the BC–alginate backbone regardless of VN loading concentration [17,18]. Notably, VN incorporation produced concentration-dependent spectral perturbations. At 0.25% VN, a distinctive band emerged at 1713 cm^{-1} , consistent with carboxyl groups from phenolic compounds. Based on published data, this band indicates the presence of carboxylic acids, likely due to the presence of phenolic and fatty acids in VN [19]. VN contains phenolic acids such as p-coumaric acid, chlorogenic acid, caffeic acid, and 2,4-dihydroxybenzoic acid [20]. At this concentration, the O–H band also downshifted to 3270 cm^{-1} , reflecting increased hydrogen bond rearrangements.

Together, these findings reveal a non-linear interaction pattern, showing minor perturbations at low VN loadings, maximal structural alterations at 0.5%, and near-baseline features at 1%. This trend implies that 0.75% VN represents the optimal incorporation point, corroborating earlier thickness data, which also showed instability beyond this threshold. From an industrial perspective, films loaded with intermediate VN concentrations may offer the greatest functional synergy between extract bioactivity and polymer stability, whereas excessive loading risks reduced homogeneity and compromised mechanical performance.

Table 5. FTIR spectra of bacterial cellulose–alginate films containing different concentrations of VN extract

Functional Group (cm^{-1})	Control	0.25% VN	0.5% VN
Hydroxyl (O–H)	3370.9	3270.7	3271.3
Alkane (C–H)	2942.7	2885.7, 2942.6	2884.9, 2943.6
Amide I (NH, C=O)	1654.3	1645.8	1645.2
Carboxylic Acid (COOH)	N/A	1713.	N/A
Aromatic (C=C)	1419.5	1410.5	1410.9
Glycosidic (C–O–C)	994.1, 925.1, 1112.5, 1043.3	923.2, 1108.6, 1032.9	923.8, 1109.6, 1034.0

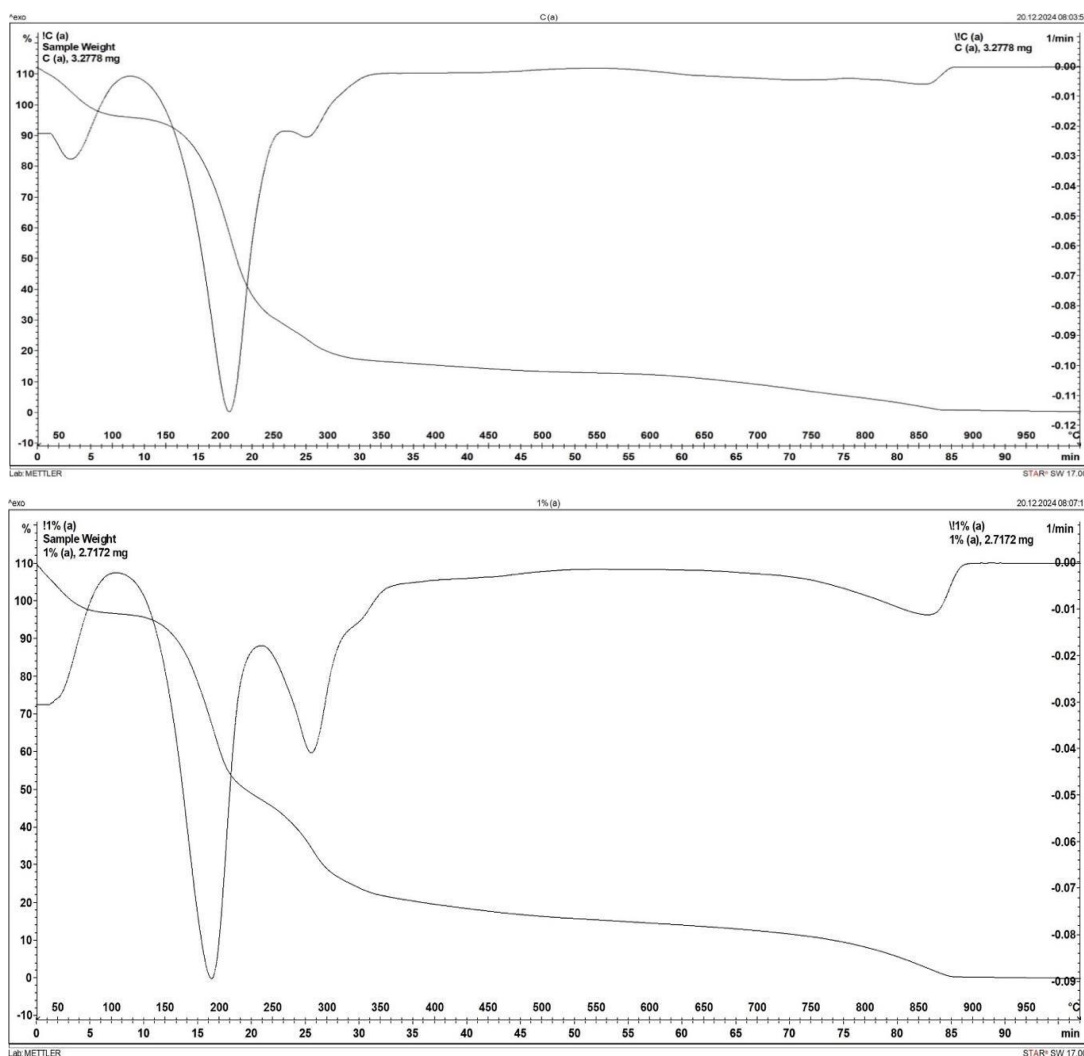


Figure 4. TGA graph of the film: (a) control film, and (b) ABV film

3.5 Thermogravimetric Analysis

TGA analysis helps ensure the suitability, performance, and quality of the film packaging throughout production and storage. In the packaging sector, this helps with material selection, process optimisation, and quality assurance. Selecting packaging materials that can withstand processes such as extrusion and storage conditions without appreciable deterioration at high temperatures requires an understanding of the thermal stability of film packaging [21]. The TGA curve of the control film (Figure 4(a)) shows two main phases of considerable weight loss. The first weight loss below 150°C is caused by the evaporation of volatile components and moisture. The breakdown of the material's organic components was indicated by significant decomposition between 250°C and 320°C. Following that, at 350–850°C, the sample's low residual weight suggests that most of it thermally decomposed, leaving little residue. Compared with the control, the ABV film (Figure 4(b)) exhibited similar thermal stability, with the initial weight loss due to moisture evaporation occurring within a comparable temperature range. This similarity indicates that incorporating VN did not reduce matrix stability. Although the additional degradation events were observed in ABV films in comparison to those of the control film, it was indirectly confirmed that the incorporation of VN extract into the matrix. The results showed that the VN extract-incorporated film is stable at body temperature, indicating its suitability for use as a wound-healing patch. Additionally, the VN extract did not adversely affect the film's thermal stability. The successful incorporation of bioactive compounds from VN extract suggests the potential of the film for use as an active packaging material that could provide antioxidant, antimicrobial, or UV-protective activity.

4. Conclusions

This research establishes that adding VN extract to alginate–bacterial cellulose films induces concentration-dependent changes in their physicochemical and functional profiles. Overall, the highest VN incorporation (0.5%) yields an optimal balance among structural stability, molecular compatibility, and antioxidant efficacy. This work contributes to the expanding field of bioactive biopolymer composites by highlighting concentration as a pivotal parameter for achieving synergistic material properties. Future studies should investigate mechanical strength, bioactive release kinetics, and production scalability to support real-world applications.

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Declaration of Competing Interests

Conflict of interest: The authors declare no conflict of interest.

CRedit Author Contribution Statement

P.D. Ponselvarajah: Methodology, Analysis, Visualisation, Writing- Original draft

P.A. Azli: Methodology, Investigation, Validation, Writing- Original draft

L.K. Murugan: Methodology, Investigation, Validation, Writing- Original draft

N. Mahmud: Conceptualisation, Methodology, Writing- Review and Editing, Supervision

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Ethics Approval

This study did not involve human participants or animals. Ethics approval was therefore not required.

Declaration of Generative AI in Scientific Writing

During the preparation of this work, the authors used AI to improve sentence structure and grammar. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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