Evaluation in Cellulose Nanocrystals Effectiveness on Composite Film based Wound Dressing from Poly(vinyl alcohol) and Gum Tragacanth

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Abstract

Biomaterial-based wound dressings were fabricated using cellulose nanocrystals (CNCs) as nano-filler in a polymeric mixture of poly(vinyl alcohol) (PVA) and gum tragacanth (GT) via solution casting. Physical and chemical characteristics of neat PVA, PVA/GT and PVA/GT/CNC films with varying concentrations (2 to 10%) of CNCs were observed. Initial analysis of CNCs showed nanosized particles of 104 nm length and 7 nm width. Scanning electron microscopy (SEM) illustrated cluster formations of CNCs in the polymer matrix. Fourier transform infrared (FTIR) spectrometry was used to confirm the chemical functional groups in the material. The presence of GT and CNCs in the polymer matrix improved water uptake and prolonged stability for 7 days. The CNCs enhanced tensile strength from 54.63 MPa to 80.39MPa. Biological properties of PVA/GT/CNC films were analyzed. Results showed that the dressing material was nontoxic to mouse fibroblast cells L929, while film loaded with betel leaf extract exhibited excellent antibacterial activities against *Staphylococcus aureus* DMST 8840 and *Pseudomonas aeruginosa* TISTR 781, indicating that composite film was suitable for application in wound dressing.

Introduction

Wound dressing involves complex biochemical and cellular processes as well as associated pathological conditions (Dhivya et al., 2015; Gupta et al., 2019). Different treatment strategies and wound dressings with special properties are required to regenerate damaged tissues. Wound dressing material should fully cover the affected area, contribute a moist environment to prevent contamination and have the ability to absorb exudates on the wound surface. Medicated dressings can degrade rapidly and they must be changed frequently and easily without causing pain or trauma at the wound bed; they must also be non-toxic, and cost effective (Ilenghoven et al., 2017; Jun et al., 2019). Film dressings are commonly applied for superficial injuries to protect skin prone to abrasion or external contamination (Jun et al., 2019). Film transparency allows daily observation without dressing removal and prevents damage to the wound bed (Ilenghoven et al., 2017). Recent advances in polymer science have led to novel designs of composite materials with unique properties for numerous biomedical applications such as wound dressings.

Natural and synthetic polymers and blended composite materials have been previously investigated. Poly(vinyl alcohol) (PVA) is a linear synthetic polymer with nontoxic, biocompatible, thermostable, water soluble and film forming properties (Xu et al., 2020). PVA has been used as the main matrix with outstanding physical and chemical properties in biomedical applications (Aslam et al., 2018; Teodorescu et al., 2019). However, the scope of PVA application is limited by its instability, insufficient elasticity and lack of cell-specific bioactivities (Teodorescu et al., 2019; Yadav et al., 2017). Several attempts have been made to improve PVA properties by incorporating substances such as chitosan (Lin et al., 2019; Zhang et al., 2015), dextran (Zheng et al., 2019), poly(ion liquid) (Fang et al., 2019) and nanocellulose (Choo et al., 2016; Wahid et al., 2019). PVA-based wound dressings were also reported including PVA composite films containing bacterial cellulose and epsilon poly-lysine (Wahid et al., 2019), PVA and dextran aldehyde hydrogel (Zheng et al., 2019) and silk fibroin-PVA composite film coated with chitosan-ZnO nanoparticles (Patil et al., 2019). These dressings demonstrated good absorb ability, tissue regeneration with low

cytotoxicity and inhibited bacterial infections with enhanced mechanical properties but the dressing materials lacked essential features such as good swelling properties and mediated angiogenesis.

Gum tragacanth (GT) is one of the most abundant and renewable natural raw materials. GT is readily accessible, relatively affordable, non-toxic, biocompatible, environmentally friendly and widely used in biomedical science (Taghavizadeh Yazdi et al., 2021) as a natural polysaccharide gum applied in various biotechnological industries (Ahmad et al., 2019). GT comprises a complex mixture of highly branched heterogeneous hydrophilic polysaccharides as mainly D-galacturonic acid and other sugar units with numerous functional groups that provide a suitable medium for cell growth and mediated angiogenesis (Ahmad et al., 2019; Nazarzadeh Zare et al., 2019; Ranjbar-Mohammadi et al., 2016). GT has been incorporated into PVA in applications such as drug delivery (Abdoli et al., 2020), scaffold (Ranjbar Mohammadi et al., 2020; Zarekhalili et al., 2017) and wound dressing (Ranjbar-Mohammadi et al., 2013; B. Singh et al., 2016).

Cellulose nanocrystals (CNCs) show potential as reinforced fillers with needle-like shapes ranging 100-250 nm in length and 5-70 nm in width, with high aspect ratio and high crystallinity (Khoo et al., 2018). Dispersion of CNCs in polymers will provides good mechanical properties due to their unique crystalline regions with inherent stability that contributes stiffness and elasticity to the material (Du et al., 2019). CNCs have zero or low toxicity and can stimulate long-term cell proliferation (Seabra et al., 2018). Hence, CNCs have been applied in various aspects of medical applications. Antimicrobial activity is also an important factor for wound dressing materials, and prevents contamination or bacterial infection while improving the healing process. Plant-based bioactive compounds are widely used an antibacterial agent in wound dressings (Avila-Salas et al., 2019; Gaspar-Pintiliescu et al., 2019). Betel leaf (*Piper betle* L.) is a traditional herbal medicine commonly found in Southeast Asia and East African countries (M. Madhumita et al., 2020; Umar et al., 2018) that contains phenolics, ethanolics, flavonoids, alcohols, alkaloids, terpenes, fatty acids and organic acids. These compounds exhibit several biological antibacterial, antifungal, anti-inflammatory and antioxidant activities (Datta et al., 2011; Dwivedi and Tripathi, 2014; M. Madhumita et al., 2020; Taukoora et al., 2016).

Here, PVA, GT and CNCs were utilized to produce biomaterial-based wound dressings. Biological properties of PVA/GT/CNC were examined for the film's ability to promote cell growth and prevent bacterial infection by loading crude betel leaf extract as an antimicrobial agent. Physicochemical characteristics of PVA/GT/CNC films were evaluated by scanning electron microscopy, transmission electron microscopy, mechanical tests and swelling capacity.

Experimental

Nanocellulose extraction from sugarcane bagasse (SCB)

SCB was dried at 55 °C for 24 h and then treated by steam explosion (Nitto Koatsu, Japan) at 190 °C with a pressure of 13 MPa for 15 min (Rocha et al., 2012). The exploded sample without sugar-rich liquid

fraction was continuously treated with 1.4% w/w NaClO₂ in diluted CH₃COOH at 70 °C. Bleaching chemicals were added every hour until the sample turned white. The bleached sample was then filtered and washed several times until the pH was neutral (N. T. Lam, W. Saewong, et al., 2017; Sukyai et al., 2018). Dried bleached cellulose fibers were dispersed in 64% w/v H₂SO₄ at 1:20 solid-liquid ratio with constant stirring 500 rpm for 75 min at 45 °C. The hydrolysis reaction was stopped using cold deionized (DI) water and repeated centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatant was removed and replaced by clean DI water, followed by dialysis. Afterward, the CNCs were sonicated (Bransonic Model 2201R-MT, USA) and kept at 4 °C until required for further use.

Preparation of composite films

An aqueous solution of PVA (10% w/v) was prepared under continuous stirring at 80 °C, and 10% w/w of GT based on a specific amount of PVA was added to create PVA/GT solution (Yadav et al., 2017). CNC composite films were fabricated by adding CNC suspension varying from 2–10% to PVA/GT solution, denoted as PVA/GT/CNC2 to PVA/GT/CNC10. The mixtures were stirred for 30 min to obtain a homogenous state before pouring into Petri dishes and drying at 37 °C for 48 h. The dried films were soaked in crude betel leaf extract prepared in propylene glycol (PG) at different concentrations (2%, 3% and 4%) for 24 h and dried at 37 °C as the antimicrobial agent.

Transmission electron microscopy (TEM)

CNC particle dimensions were examined by TEM. Briefly, 0.01% w/v CNC suspension was deposited on a carbon-coated copper grid, post-stained with 2% w/v uranyl acetate solution and dried for 8 min. The sample was analyzed under TEM (Hitachi Model HT7700, Japan) with an accelerating voltage of 100 kV. CNC dimensions were determined using the ImageJ program.

Optical and transparency measurement

Film transparency was determined using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 560 nm (Vanitjinda et al., 2019). Film specimens were cut into rectangular shapes (2 x 40 mm) and placed inside the spectrophotometer cells. An empty spectrophotometer cell was set as a blank. All measurements were conducted in triplicate. The percentage of film transparency was calculated using the formula (1) (Mostafavi et al., 2016):

$$\%T = (T_f / T_b) \times 100 \quad (1)$$

where T_f and T_b are the transmittance values of the film sample and blank cell, respectively.

Scanning electron microscopy (SEM)

Surface and cross section of the composite films were investigated by SEM (Hitachi Model, Joel JSM5600LV, Japan) with an accelerating voltage of 15-20 kV. To observe the cross section, film specimens were freeze-cracked following immersion in liquid nitrogen. Each piece was deposited on a cylindrical holder and coated with a thin gold layer before observation.

Fourier transform infrared (FTIR) spectrometry analysis

PVA, PVA/GT and PVA/GT/CNC composite films were cut into 10 x 10 mm squares at random locations. Infrared spectra of each sample were recorded using an FTIR (Bruker Tensor 27 Spectrometer, USA) in the range of 4000-500 cm^{-1} with a resolution of 4 cm^{-1} .

Swelling ratio and stability

Swelling ratio and stability of the composite films were evaluated based on the amount of water absorbed and percentage of weight loss after 7 days, respectively. Briefly, dried film specimens (15 x 15 mm) were immersed in DI water at ambient temperature. For each turn of measurement (Popescu et al., 2017), swelling ratio was assessed by the weight of swollen samples. To examine the stability of composite films, swollen samples were taken and dried again to measure the difference in dried weight before and after immersion. All samples were weighed by an analytical balance with three replications and results were calculated using the formulae (2) and (3) (Zheng et al., 2019):

$$\text{Swelling ratio} = (M_1 - M_0)/M_0 \times 100 \quad (2)$$

$$\text{Weight loss} = (M_0 - M_1)/M_0 \times 100 \quad (3)$$

where M_0 and M_1 represent sample weight before and after immersion, respectively.

Mechanical properties

Tensile strength and elongation at break of the composite films were evaluated using a universal testing machine (AGS-J 1kN, Japan) according to ASTM D882-02 Standard Method. Briefly, films were cut into rectangular shapes (10 x 50 mm) and kept at 25 °C with relative humidity of 50% $\pm$ 2% until reaching constant weight. The films were tightly fixed in the grips with 30 mm initial space and pulled apart by a 1 kN load cell. The experiment was repeated as three replications, and tensile strength and elongation at break were calculated from the formulae (4) and (5), respectively (Mostafavi et al., 2016):

$$\text{Tensile strength} = F/(T \times W) \quad (4)$$

where F , T and X are the maximum force, film thickness and width of film, respectively.

$$\text{Elongation at break} = (D_1 - D_0) \times 100 \quad (5)$$

where D_1 is the distance of rupture and D_0 is the initial distance between grips.

Cytotoxicity

Cytotoxicity testing was conducted using the colorimetric MTT assay of tetrazolium dye that evaluated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide following the on ISO 10993-5 standard (Simon, 2004). Mouse fibroblast cells L929 (NCTC clone 929:CCL1 from the American Type Culture Collection (ATCC), strain L) were cultured in Minimum Essential Medium (MEM) with an appropriate cell density of

10^5 cells/ml and incubated for 24 ± 2 h. The films were sterilized by UV irradiation for 15 min, prepared at 3 cm²/ml and extracted for 24 ± 2 h at 37 ± 1 °C. The extracts were further incubated for 24 ± 2 h before being stained with MTT assay for 2 h. A Thermanox (Nunc) coverslip was used as the negative control, while polyurethane film containing 0.1% zinc diethyldithiocarbamate served as the positive control. A culturing with fibroblast cells for 24 h, tested samples were compared to the negative and positive controls. A microplate reader was used to determine cell viability at absorbance of 570 nm using equation (6) (Ranjbar-Mohammadi et al., 2013). Cell viability above 70% was considered non-cytotoxic.

$$\text{Cell viability} = \text{OD}_{\text{sample}} / \text{OD}_{\text{blank}} \times 100\% \quad (6)$$

where $\text{OD}_{\text{sample}}$ and OD_{blank} are measurements of optical density of the test sample and blank sample, respectively.

Antimicrobial ability

Antimicrobial properties of films loaded with crude betel leaf extract were studied against *Staphylococcus aureus* DMST 8840 and *Pseudomonas aeruginosa* TISTR 781 using the agar diffusion method. Before the experiment, *S. aureus* and *P. aeruginosa* suspensions of 10^6 CFU/ml were evenly spread over nutrient agar plates. Films loaded with betel leaf extract were placed on an inoculated agar surface, with paper discs loaded with erythromycin used as the positive control and PG as the negative control. The culture plates were incubated at 37 °C for 18 h, and the inhibition zone was measured at the end of the incubation time.

Statistical analysis

Statistical analysis of the experimental data was performed by one-way analysis of variance (ANOVA) at a confidence level of 0.05 using the Minitab program. All data were expressed as mean $\pm$ standard deviation. A p-value < 0.05 was considered statistically significant.

Results And Discussion

SCB (Fig. 1a) consists of cellulosic and non-cellulosic components. A series of treatment steps were conducted to remove the non-cellulosic components (hemicellulose and lignin) to promote defibrillation. Removal of major non-cellulosic components increased the cellulose content. The bleached fiber (Fig. 1b) was subsequently applied in acid hydrolysis that caused disordering in the glycosidic linkages and breakdown of the fibrous structure, resulted in reduction of fiber size of CNCs (Tong et al., 2017). The crystalline CNCs were presented as a stable colloidal suspension (Fig. 1c). The surface of CNCs was linked to negatively charged particles of sulfate half ester groups derived from the acid hydrolysis process (Du et al., 2019). TEM analysis was carried out to determine particle size and distribution of CNCs. Results showed needle-like shapes of individual and aggregated particles (Fig. 1d). Dimensions of the CNCs were approximately 104 nm in length and 7 nm in width, similar to sizes reported earlier (N. Lam et al., 2017; Meesupthong et al., 2020).

Results in Table 1 show the percentage transparency of the composite films. Neat PVA recorded the highest transmittance of $91.27 \pm 0.43\%$ followed by PVA/GT at $74.59 \pm 0.43\%$. The PVA/GT/CNC10 film recorded the lowest transmittance of $46.28 \pm 0.54\%$. High significant difference ($p < 0.05$) was recorded between PVA, PVA/GT and PVA/GT/CNC composite films due to differences in light dispersion caused by the disparate viscosities of GT and PVA (Silva et al., 2016) and aggregation of CNCs as the concentration increased (Chen et al., 2020). Optical properties also related the re-arrangements in the internal structure of PVA molecules during the drying process (Silva et al., 2016; Tonyali et al., 2018). Opaqueness of the films increased at higher CNC concentration (Table 1) although visual observations of composite films were recorded as colorless and transparent (Fig. 2). Films with the lowest and highest concentration of CNCs were selected for SEM analysis.

Table 1
Transparency of composite film.

Samples	Transmittance
PVA	91.27 ± 0.43^b
PVA/GT	74.59 ± 0.43^c
PVA/GT/CNC2	74.30 ± 0.17^c
PVA/GT/CNC4	68.94 ± 0.11^d
PVA/GT/CNC6	68.79 ± 0.11^d
PVA/GT/CNC8	59.70 ± 0.39^e
PVA/GT/CNC10	46.28 ± 0.54^f
Control (Blank)	100 ± 0.00^a
Values with the same letter, the difference is not statistically significant, whiles different letters are statistically significant ($p \leq 0.05$).	

SEM micrographs showed the surface and cross section of PVA, PVA/GT and PVA/GT/CNC films (Fig. 3). Neat PVA film had a uniform texture with a smooth planer surface and cross section (Fig. 3a **and e**). However, when GT was added, the film surface became slightly rougher, characterized by the presence of white dots on the surface and cracks in the cross section (Fig. 3b **and f**). Mostafavi et al. (2016) reported that the chemical structure of GT could organize into a more open and porous network. Similarly, blended film containing GT showed reduced homogeneous quality (Khodaei et al., 2019; Tonyali et al., 2018) support swelling capacity. Dispersion of nanocellulose in the PVA/GT films (Fig. 3c) resulted in the disappearance of some white dots on the surface structure, suggesting that CNC particles filled in the polymeric matrix. Increasing the CNCs content led to assemblage and cluster formation of CNCs in the film (Fig. 3d). This result concurred with Jahan et al. (2018). In addition, CNC distribution was observed

on the fractured shapes in the cross section (Fig. 3g **and** h). The nanocellulose reinforced polymer network formed aggregates with a wide range of sizes and shapes in random directions (Mandal and Chakrabarty, 2015). Higher concentrations of nanocellulose induced brittle fracture because the aggregates were able to concentrate force at localized points, thus impacting mechanical properties.

Figure 4 shows the FTIR analysis of chemical functional groups of GT powder, PVA, PVA/GT and PVA/GT/CNC films. The FTIR spectra for GT powder with an absorption band at 2149 cm^{-1} corresponded to various carbonyl groups in the gum while peaks of carbonyl stretching in aldehydes, ketones and carboxylic acids were presented at 1750 cm^{-1} (Kurt, 2018). The bands at 1635 cm^{-1} and 1442 cm^{-1} were attributed to asymmetrical and symmetrical stretching of carboxylate groups, respectively, while peaks at 1242 cm^{-1} and 1020 cm^{-1} displayed C-O stretching vibration in polyols and alcoholic groups, respectively (Abdoli et al., 2020; Ranjbar-Mohammadi et al., 2013; Zarekhalili et al., 2017). The band observed in all samples at 3285 cm^{-1} was characteristic of O-H stretching groups from intra- and intermolecular hydrogen bonds (Choo et al., 2016; Jahan et al., 2018) while a wider band of O-H stretching in the GT structure observed at 3420 cm^{-1} was caused by OH and COOH groups (Mostafavi et al., 2016; Ranjbar-Mohammadi et al., 2013). Asymmetrical and symmetrical stretching vibrations of methylene groups were presented at 2939 cm^{-1} and 2908 cm^{-1} , respectively (Zarekhalili et al., 2017) while the peak at 1086 cm^{-1} was assigned to C-O stretching (Jahan et al., 2018; Mostafavi et al., 2016). Vinyl C-H in plane bending of PVA was confirmed at 1419 cm^{-1} . Furthermore, the absorption band centered at 842 cm^{-1} represented C=C bending (Abureesh et al., 2016; Mandal and Chakrabarty, 2015). No significant change in PVA matrix was recorded after the incorporation of GT and CNCs, with no effect on PVA molecule structure and so its chemical structures were maintained (S. Singh et al., 2018). Notably, addition of CNCs at high concentration contributed C=C stretching at 1655 cm^{-1} (Nandiyanto et al., 2019).

Swelling of wound dressing is an important factor that relates to wound exudates absorption and prevention of infection and can be explained by physical and chemical changes in material structure that help water molecules to diffuse internally, leading to an increase in free volume (Jahan et al., 2018; Kumar et al., 2014; Sadat Hosseini et al., 2016). Results in Fig. 5 showed the swelling ability of the various films. Initially, the large numbers of free hydroxyl groups in PVA film absorbed water molecules and reached the maximum state of approximately 260% before reducing at 1.5 h. PVA/GT films increased gradually and then remained at a steady rate of 250% due to the presence of hydrogen bonds in hydroxyl and carboxyl functional groups (Kurt, 2018). The porous structure of GT trapped water molecules (Khodaei et al., 2019; Nazarzadeh Zare et al., 2019; Rao et al., 2017). After 6 h, water content slightly decreased to 230% up to 7 days due to degradation in some linking points in the polymer matrix (Tavakol et al., 2014). There were no obvious differences in PVA/GT/CNC2 and PVA/GT films. Addition of small amounts of CNCs increased water absorption in the polymer matrix. However, as CNC concentration increased, extra swelling capacity was restrained (Jahan et al., 2018; Popescu et al., 2017; Sutka et al., 2015) due to the reinforcing effects of CNCs. This reduction illustrated that hydrogen bonding between CNC groups. Thus, water molecules could not freely pass through the polymer (Slavutsky and Bertuzzi, 2014; Sutka et al., 2015). The PVA/GT/CNC2 film provided swelling behavior that could create an

environment suitable for wound healing (Fang et al., 2019; Patil et al., 2019; Sadat Hosseini et al., 2016; Zhao et al., 2017).

Stability of the wound dressing was assessed as the percentage weight loss of composite film to indicate prolonged use (Zheng et al., 2019). Results in Fig. 6 showed the PVA films exhibited the highest rate of weight loss of 9.8% on the first day, and this increased to 17.2% on the 7th day due to solvation and fragmentation of the film (Kumar et al., 2014). However, addition of GT improved film stability by maintaining weight loss below 12% after 7 days. Bassorin fragments in GT are insoluble in water, thus reducing the film solubility (Pirsa et al., 2020). Solubility was further reduced after 7 days when CNCs were added due to the formation of a strong matrix of hydrogen bonds through the three-dimensional structure of CNCs and the polymeric matrix that reduced free hydroxyl groups and restricted water penetration (Popescu et al., 2017; Slavutsky and Bertuzzi, 2014).

Mechanical properties of the films are summarized in Table 2. Wound dressing should be strong, flexible and elastic for efficient treatment; thus, the film was evaluated in terms of tensile strength, elongation at break and elastic modulus (Tong et al., 2017). Neat PVA film exhibited tensile strength of 54.63 MPa, which reduced slightly to 49.26 MPa when GT was added. There was no significant difference ($p < 0.05$) between neat PVA and PVA/GT. This result concurred with by Ojagh et al. (2017) who found that addition of GT had no significant effect on mechanical properties. Highest tensile strength was recorded by PVA/GT/CNC2 (80.39MPa), while PVA/GT/CNC6 gave the lowest strength of 45.05 ± 3.39 MPa. Small amounts of nanocellulose increased the strength of the material by entrapping nano-filler inside the matrix. This allowed strong hydrogen bond formation between nanocellulose and matrix, thus imparting mechanical integrity (N. Lam et al., 2017; Sun et al., 2017). However, high concentration of CNCs led to agglomeration of particles, increased rigidity and poor distribution in polymer matrices, thus impacting the formation of hydrogen bonds among polymer chains and inhibiting reinforcing properties (Choo et al., 2016; Mandal and Chakrabarty, 2015). Elastic modulus values of PVA and PVA/GT were 1223.08 ± 182.08 MPa and 1062.51 ± 101.65 MPa, respectively. Highest elastic modulus of 1526.11 ± 31.86 MPa was recorded by PVA/GT/CNC2 film, which decreased to 1260.45 ± 76.94 MPa in PVA/GT/CNC10 film. The high value was attributed to the crystalline nature of CNCs that resulted in better alignment and enhanced elastic modulus (Jahan et al., 2018; Sun et al., 2017). Although there was no significant difference in elastic modulus, composite materials loaded with CNCs showed higher values than neat PVA and PVA/GT samples. Mechanical properties of wound dressing were recommended to cover the mechanical properties of human skin (5-30 MPa) depending on body region (Bombaldi de Souza et al., 2020). Improvement in tensile strength should be adequate for application and storage, to ensure that the dressing is not be damaged by handling (Elsner et al., 2012; Pires and Moraes, 2014). Therefore, PVA/GT/CNC composite materials in the range of 45-80 MPa showed good mechanical properties compared to previous studies (Pires and Moraes, 2014; Schoeler et al., 2020; Tong et al., 2017). Neat PVA had elongation at break of 48.52% and this slightly decreased to 44.48% in PVA/GT, while elongation at break of PVA/GT/CNC films decreased with increasing concentration of CNCs. The elongation at break significantly decreased to 8.11% in PVA/GT/CNC2 and continuously dropped to 3.92% in PVA/GT/CNC10 film due to the rigid nature of CNCs. The rough structure with cracks inside the polymer matrix (Fig. 3h)

led to the material being easily broken easily under pressure (Mandal and Chakrabarty, 2015). Since CNCs are not deformable, strong interaction between CNCs and the matrix did not allow elongation in the composite materials (Ching et al., 2015).

Table 2
Mechanical properties of composite films.

Sample	Tensile strength (MPa)	Elongation at break (%)	Elastic modulus (MPa)
PVA	54.63 ± 0.69 ^{bc}	48.52 ± 1.57 ^a	1223.08 ± 182.08 ^a
PVA/GT	49.26 ± 5.00 ^{bc}	44.38 ± 5.03 ^a	1062.51 ± 101.65 ^a
PVA/GT/CNC2	80.39 ± 1.41 ^a	8.11 ± 0.30 ^b	1526.11 ± 31.86 ^a
PVA/GT/CNC4	59.09 ± 4.26 ^b	7.36 ± 0.82 ^b	1450.74 ± 76.94 ^a
PVA/GT/CNC6	45.05 ± 3.39 ^c	8.81 ± 1.56 ^b	1299.13 ± 120.37 ^a
PVA/GT/CNC8	47.63 ± 3.54 ^{bc}	6.88 ± 0.06 ^b	1353.38 ± 177.19 ^a
PVA/GT/CNC10	47.68 ± 9.26 ^{bc}	3.92 ± 0.92 ^b	1260.45 ± 76.94 ^a
Values with the same letter, the difference is not statistically significant, while different letters are statistically significant ($p \leq 0.05$).			

The cytotoxicities of PVA, PVA/GT, and PVA/GT/CNC10 films were evaluated using the MTT assay. The sample with the highest CNC concentration (PVA/GT/CNC10) was tested to confirm non-toxicity and safety of the film for cell growth. Results showed significant differences between test samples and the positive control, while there were no significant differences between the test samples and the negative control (Fig. 8). Cell viabilities of PVA and PVA/GT were 93% and 84%, respectively. Decrease in cell viability of PVA/GT film may be due to the diversity in chemical composition that slightly influenced the biological properties (Nazarzadeh Zare et al., 2019). The high concentration of CNCs was found to be non-toxic to fibroblast cells with 95% cell viability. Incorporation of CNCs in the polymeric matrix and reaction between CNCs and PVA/GT improved cell viability in the films. All samples were non-toxic to fibroblast cells and satisfied non-cytotoxic requirement of cell viability above 70%, in agreement with results reported by previous studies (N. T. Lam, R. Chollakup, et al., 2017; Zarekhalili et al., 2017).

Disc diffusion results revealed that films loaded with betel leaf extract at different concentrations of 2%, 3% and 4%, exhibited excellent antibacterial activity against both gram-negative (*P. aeruginosa*) and gram-positive (*S. aureus*) microorganisms. According to Othman et al. (2018), antibacterial activity of betel leaf extract started to be observed at 100 mg/ml. Results in Fig. 9 show clear zones around discs, in agreement with previous studies (Akter et al., 2014; Mitali Madhumita et al., 2019; Taukoorah et al., 2016). The inhibition zone increased with increasing concentration of betel leaf extract. The susceptibility of *P. aeruginosa* was less than *S. aureus* (Table 3) due to differences in their morphology. The essential oil

contained in betel leaf extract inhibited bacterial cell growth due to hydroxyl groups in the phenolic compounds and the hydrophobicity of fatty acids and hydroxyl fatty acid ester components. These groups caused destabilization on the cytoplasmic membrane disrupted proton and electron flow and discharge ATP/decreased ATP synthesis leading to cell death (Nouri and Mohammadi Nafchi, 2014; Umar et al., 2018). The zone of inhibition of *P. aeruginosa* was less than *S. aureus* due to the outer layer of phospholipidic membrane that contributed an impermeable strong cell wall (Arias et al., 2004; Umar et al., 2018). The films showed higher inhibition zones than the positive control, while the negative control did not show any inhibition in both strains, proving that betel leaf extract activity was not affected by PG.

Table 3
Antimicrobial activity of PVA/GT/CNC2 film loaded betel extract on agar diffusion test.

Sample	Diameter of inhibition zone (mm)	
	<i>P. aeruginosa</i>	<i>S. aureus</i>
Betel 2%	16.60 ± 3.21 ^b	18.00 ± 1.87 ^c
Betel 3%	19.00 ± 1.41 ^b	22.00 ± 3.83 ^b
Betel 4%	23.60 ± 0.55 ^a	28.20 ± 0.84 ^a
Positive control	12.30 ± 0.57 ^c	14.40 ± 1.14 ^c
Negative control	-	-
Values with the same letter, the difference is not statistically significant, whiles different letters are statistically significant (p ≤ 0.05).		

Conclusions

PVA/GT/CNC composite film was successfully prepared as wound dressing material. The PVA/GT/CNC2 film exhibited higher swelling ratio and mechanical properties with good transparency. These properties could help to absorb exudates with easy daily observation, and minimize trauma to the wound bed. Apart from the physicochemical enhancements, cytocompatibility was also verified by mouse fibroblast cells that showed the film to be non-toxic. The film also exhibited excellent antibacterial activity by loading betel leaf extract against both gram-negative (*P. aeruginosa*) and gram-positive (*S. aureus*) bacteria. Composite films showed promise as candidates for healing protection in cutaneous wound dressing application.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest

Human/Animal Rights

This article does not contain any studies with human or animal subject performed by any authors

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Consent to participate

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Code availability

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Figures

Figure 1

Photographs of (a) SCB, (b) bleached fiber, (c) CNCs, and (d) TEM image of CNCs.

Figure 2

Photographs of (a) PVA, (b) PVA/GT, (c) PVA/GT/CNC2, and (d) PVA/GT/CNC10.

Figure 3

SEM micrographs of surface of (a) PVA film, (b) PVA/GT film, (c) PVA/GT/CNC2, (d) PVA/GT/CNC10, and cross section of (e) PVA film, (f) PVA/GT film, (g) PVA/GT/CNC2 and (h) PVA/GT/CNC10.

Figure 4

FTIR spectra of (a) GT, (b) PVA, (c) PVA/GT, (d) PVA/GT/CNC2, (e) PVA/GT/CNC4, (f) PVA/GT/CNC6, (g) PVA/GT/CNC8, and (h) PVA/GT/CNC10.

Figure 5

Swelling ratio of composite films for 7 days.

Figure 6

Percentage of weight loss after 7 days.

Figure 7

Stress – strain curves of PVA, PVA/GT, PVA/GT/CNC2 and PVA/GT/CNC10.

Figure 8

Cell viability of PVA, PVA/GT and PVA/GT/CNC10, comparing with positive and negative controls.

Figure 9

Antibacterial activity PVA/GT/CNC2 film loading 2%, 3% and 4% of crude betel extract against *P. aeruginosa* (left) and *S. aureus* (right).