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Antimicrobial wound dressing film utilizing nano-cellulose and gelatin as drug delivery system for *Garcinia mangostana* L extract

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ABSTRACT

Aims: Diabetic patients with foot ulcer showed 150-fold increased risk of amputation, which is primarily caused by microbial infection. Silver ions are commonly incorporated into wound dressing to enhance the antimicrobial property. However, concerns have been expressed about the development of bacterial resistance to heavy metals. In this study, we extracted the nano-cellulose from medical cotton and reinforced with gelatin to develop a film for wound dressing.
Methodology and results: *Garcinia mangostana* L pericarp extract was incorporated into the nano-cellulose film as antimicrobial finishing. The efficacy of the developed nano-cellulose film was evaluated on diabetic wound microorganisms. We observed cellulose nano crystals with an average length of 133.71 nm under transmission electron microscope. The developed film showed gradual release of the extract over a period of 48 h and no burst effect was observed. The film exhibited significant inhibitory activity on three Gram positive bacteria, three Gram negative and all filamentous fungi tested. On Hohenstein challenge test, all test microorganisms showed significant growth reduction, with the treatment of the film. We also noticed that the antimicrobial activity of the film sustained even after 20 washes.
Conclusion, significance and impact of study: Our results indicate that the *G. mangostana* L pericarp extract loaded nano-cellulose films exhibited significant inhibitory activity on diabetic wound microorganisms. The developed film can be potentially used to prevent foot ulcer infection among diabetic patients.

Keywords: Garcinia mangostana, pericarp extract, antimicrobial activity, nanocellulose, gelatin

INTRODUCTION

Diabetes mellitus is defined as a metabolic disease characterized by hyperglycemia resulting from defect in insulin action or insulin secretion (Barbagallo and Dominguez, 2007). According to World Health Organization (WHO), the number of adults with diabetes has risen from 108 million cases in 1980 to 451 million in 2017 (Cho *et al*., 2018). The figure is projected to increase, and diabetes will be the 7th leading cause of death in 2030 (Mathers and Loncar, 2006). Foot ulcer is one of the common complications among diabetic patients. These ulcers lead to necrotizing fasciitis, soft tissue gangrene, septic arthritis, and osteomyelitis in the patients (Bader, 2008).

Poor wound management of the foot ulcer in diabetic patients has caused 150-fold higher risk of amputation. The surface of wound dressing for foot ulcer is often found with high microbial load and this becomes the main cause of delayed healing time. Therefore, it is important to prevent the infection of foot ulcer in order to improve the healing process. One of the effective alternatives to prevent the infection on the wound surface is the application of antimicrobial substance-loaded film as wound dressing (Bowler *et al*., 2001).

Nano-cellulose is a nano-scaled biopolymer derived from plants or bacteria. Nano-cellulose shows good biocompatibility, biodegradability and low toxicity compared to traditional materials (Maneerung *et al*., 2008; Lin and Dufresne, 2014). Thus, nano-cellulose is an excellent candidate for wound dressing material. This is particularly attractive due to its good compaction property as well as its ability to absorb exudates during the wound healing process (Maneerung *et al*., 2008). Nano-cellulose has been applied in clinical practice for dressing of deep wound, burn wound and diabetic ulcer (Lin and Dufresne, 2014). Besides, nano-cellulose has gained attention as an

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approach to deliver bioactive compounds to further accelerate the wound healing process.

Metal especially silver or zinc ions are among the most common antimicrobial agent incorporated into the cotton material to enhance the antimicrobial activity of the wound dressing. However, there are several drawbacks of such class of antimicrobial agents. Of all, the chronic ingestion or inhalation of heavy metal can lead to deposition of heavy metal particles in the skin, eyes, kidneys and liver (Pannerselvam *et al*., 2017). Besides, there are also concerns of usage in the patients who are allergic to silver. Therefore, it is important to find an effective replacement of silver as antimicrobial agent in wound dressing.

In this study, we evaluated the efficacy of nano-cellulose film loaded with the *G. mangostana* L pericarp extract as a natural source of antimicrobial agent. *G. mangostana* (*G. mangostana* Linn., GML), a well-known tropical fruit, that is indigenous to Southeast Asia but can be found in most tropical countries. This fruit exhibits a variety of pharmacological activities (Qiang *et al*., 2017). The pericarp of the fruit contains considerable amounts of biologically active compounds, such as xanthones, terpenes, anthocyanins, tannins, and phenols. For centuries, the pericarp of *G. mangostana* was used as a medicinal agent by Southeast Asians in the treatment of skin infections and wounds, amoebic dysentery, diarrhea, and cholera (Nakamura *et al*., 2008). To date, it has not been shown to cause any toxicity effect.

MATERIALS AND METHODS

Isolation of nano-cellulose

The isolation of nano-cellulose was performed according to Tong *et al*. (2018). In order to isolate the nano-cellulose, pulping process was conducted by soaking 5 g of medical grade cotton fiber (Premiera, Malaysia) in 1 M sodium hydroxide solution at a ratio of 1:20 (w/v) at 160 °C for 2 h. The pulp was washed thoroughly with distilled water to remove excessive sodium hydroxide. Then, the cotton fibers were filtered with Whatman No 1 filter papers and dried at 50 °C until a constant weight was obtained. To remove lignin from the cotton fiber, the fibers were acidified with 30%sulfuric acid (1:20, w/v) for 6 h with continuous agitation. Refrigerated water (4 °C) was then added to stop the reaction. The cotton fibers were then sonicated in a water bath (4 °C) for 20 min at sonication power 1000 W in an ultrasonic generator. After that, the mixture was centrifuged at 3500 *g* for 15 min at room temperature. The supernatant was decanted to remove the excess water. The pellet was collected as nano cellulose fibers. The fibers were then dried at 50 °C for 72 h.

Extraction of *G. mangostana* pericarp

The pericarp of G. *mangostana* was extracted according to Satong-Aun *et al*. (2011) with modifications. The pericarp was tray-dried at 50 °C for 72 h and ground into powder using hammer mill and sieved to obtain particles diameter between 500-1000 µm (18 mesh). The fine powder (5 g) was extracted using Soxlet apparatus (Soxtec system HT2 1045, Foss Tecator, Sweden) with ethanol. Extraction was carried out for 2 h that include initial boiling for 30 min and rinsing for 2 h. The extract was collected and filtered, the filtrate was concentrated using rotatory evaporator at 40 °C. The extract was kept in airtight amber bottles after flushing with nitrogen gas for 30 sec. The extract was stored in freezer at –20 °C until further use.

Development of nano-cellusose gelatin film

The nano-cellulose gelatin film was developed according to Taokaew *et al.* (2013). Firstly, 5 g of nano-cellulose fibers was first suspended in 100 mL of 10% gelatin solution. Then, 5 mL of the pericarp extract (10 mg/mL) was added into the mixture. The mixture was then homogenized at 8000 rpm for 10 min. Then, 20 mL of the mixture was finally cast on a circular container with 90 mm diameter and left to dry at room temperature. A control film was prepared by replacing the pericarp extract with equal volume of ethanol.

Microscopic analysis

The nano cellulose suspension was observed optically using transmission electron microscopy (TEM; Philips, Model CM12, Eindhoven, Netherlands), operating at 120 kV. To prepare the sample for TEM observation, a clean dropper was used to transfer a droplet of sample on a carbon-coated copper grid. The sample was allowed to air-dry about 3 min at room temperature. Then, a small drop of uranyl acetate stain was applied to the grid. After 1 min, the excess stain was removed and the grid was examined with TEM.

Test microorganisms

The test bacteria used in this study include four Gram positive bacteria (*Bacillus cereus*, *B. coagulans*, *Streptococcus* sp. and Methicilin-resistant *Staphylococcus aureus*), four Gram negative bacteria (*Escherichia coli*, *Proteus mirabilis*, *Yersinia* sp. and *Pseudomonas aeruginosa*). The developed film was also tested for their antifungal activity on 4 filamentous fungi (*Aspergillus niger*, *Rhizopus stolonifer*, *Microsporum fulvum* and *Fusarium solani*). All the test microorganisms were previously isolated from diabetic patients in Hospital Seberang Jaya, Penang, with a cohort of chronic wounds. The test microorganisms were sub-cultured every 2 weeks in order to maintain its viability. The microbial inoculums were prepared as per protocols described by Yenn *et al*. (2014).

Agar diffusion test

The test was performed by spreading 0.1 mL of the inoculums to the surface of Mueller–Hinton agar (Merck)

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using cotton swab (Yenn *et al*., 2014). Thenano-cellusose gelatin film with *G. mangostana* L pericarp extract excised to a disc (20 mm) was then placed on the agar medium. The nano-cellulose film, without addition of *G. mangostana* L extract (with 97% ethanol) was served as negative control. The experiment was done in triplicate in separate occasions. All plates were incubated at 37 °C for 24 h. After the incubation period, the diameters of inhibition zone surrounding discs were measured.

Hohenstein challenge test (AATCC-100)

A total of 6 bacteria were selected for this test based on their susceptibility on agar diffusion test. The antimicrobial effectiveness of the nano-cellulose film was assessed according to the standard test method by American Association of Textile Chemists and Colourists (AATCC) standard (Vaideki *et al*., 2008). Firstly, 100 µL of bacterial inoculum was inoculated to 25 mL of sterile nutrient broth. The final inoculum concentration was $4×10^5$ CFU/mL. Then, 0.1 g of the film was added into the culture. All the flasks were incubated at 37 °C, with a rotational speed of 120 rpm for 24 h. After the incubation period, viable cell count was performed by inoculating the diluted sample on nutrient agar (Merck). The antimicrobial efficiency of the sample in term of percentage of growth was determined relative to control (nano-cellulose film without pericarp extract).

Wash durability test (AATCC-147)

The wash durability of the films was evaluated after different wash cycles. The sample was washed with 1% detergent solution (Breeze, Malaysia) for 5 min. The films were then rinsed with distilled water to remove any remaining detergent. The antibacterial efficacy of the wash film was subjected to Hohenstein challenge test as mentioned above, after 10 and 20 launderings.

***In vitro* release test**

The assay was performed by inserting the developed film loaded with *G. mangostana* L pericarp extract into the conical flasks containing artificial sweat solution (sodium chloride 5 g/L, urea 1 g/L, lactic acid 1 g/L; adjusted to pH 5.5 with 1 M sodium hydroxide) at a ratio of 1:100 (w/v). All the samples were placed at 37 °C, on an orbital shaker at 80 rpm. At the predetermined time point (0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72 h), a flask was withdrawn, and the absorbance was taken at 320 nm with spectrophotometer (Shidmazu). *Garcinia mangostana* extracts contain high concentration of α-mangostin, and hence this compound was selected as a reference compound (Aisha *et al*., 2013). The α-mangostin released from the film was determined based on a standard curve developed using α-Mangostin standard (Sigma). A control of the nano-cellulose film without the *G. mangostana* L pericarp extract was used.

RESULTS AND DISCUSSION

Acid treatment is commonly used to break down cellulosic microfibers into nanofibers. In this study, we utilized sulfuric acid to hydrolyze medical grade cellulosic microfibers by cleaving the amorphous regions of the microfibers (Corrêa *et al*., 2010). This causes the reduction in size of the fibers to nanoscale. Besides, the nanofibers were thoroughly sonicated to prevent the aggregation of the nanofibers (Lu and Hsieh, 2010). Under TEM micrograph (Figure 1), plenty cellulosic nanofibers were observed. The average size of the nanofibers was 133.71 nm in length, and 2 nm in width. The observations were in agreement with Dong and Roman (2007). Besides that, we also observed that the nanofibers were rod in shape, with no apparent defect. This indicates that the method used was suitable to produce cellulosic nanofibers from medical grade cotton. Aggregation of cellulose nanofibers was not observed. The visual observation of the micrograph showed significant morphological similarities with cellulose nanocrystal reported by Neto *et al*. (2013).

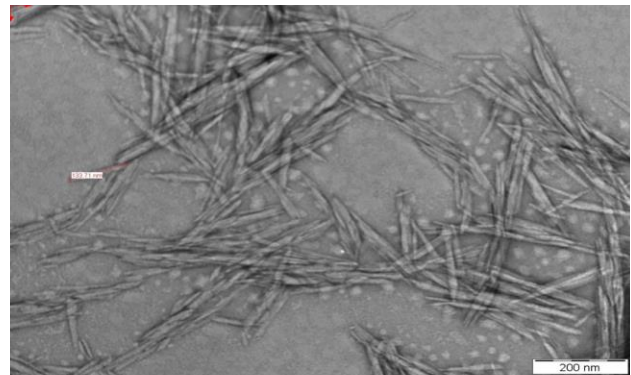

Figure 1: TEM micrograph of the nanocellulose fibers. The average size of the nanofibers was 133.71 nm in length, and 2 nm in width.

Gelatin is a collagen derivative. It has been widely used as wound dressing material because it is highly hycrophilic and has good gas barrier properties (Taokaew *et al*., 2013). Besides, it also showed good biocompatibility, thermal stability and non-toxic (Moritz *et al*., 2014). In this study, gelatin was added as polymer matrix to improve the physical and mechanical properties of the film, by acting as cross-linking agent. Polymer matrix also added to sustain the drug release from the film. Gelatin has been used as polymer matrix for wound dressing as reported by Moritz *et al*. (2014) and Shankar *et al*. (2016).

The pericarps extract loaded nano-cellulose film showed broad spectrum antimicrobial activity. The film exhibited significant inhibitory activity on three Gram positive and three Gram negative bacteria (Table 1). A negative control was included by using the nano-cellulose film without *G. mangostana* pericarps extract finishing. The results showed that the control films did not exhibit

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any inhibitory effect on all test microorganisms. The antimicrobial activity of the developed nano-cellulose film was indicated by the clear zone surrounding the films. The largest zone was observed on *Streptococcus* sp. The observation of this study is in agreement with Fernando and Dasanayaka (2006) that the crude as well as the fractionated extracts of the *G. mangostana* pericarps showed significant bactericidal effect on the growth of *Streptococcus* sp. Moreover, *S. pyogenes* was isolated in 10.6% of the diabetic foot ulcers and is the predominant pathogen of the wound (Citron *et al*., 2007).

Table 1: The antimicrobial activity of the developed films on agar diffusion test. The film with the *G. mangostana* L pericarp extract inhibited both Gram positive and Gram-negative bacteria.

Test bacteria	Antimicrobial activity	
	Pericarp extract loaded film	Negative control
Gram positive bacteria		
MRSA	+	-
Streptococcus sp.	+++	-
B. coagulans	-	-
B. cereus	++	-
Gram negative bacteria		
E. coli	+	-
P. mirabilis	-	-
Yersinia sp.	++	-
P. aeruginosa	+++	-

-: No inhibition, +: Diameter of inhibition zone >15 mm, ++: Diameter of inhibition zone >20mm, +++: Diameter of inhibition zone>25mm

In the antifungal susceptibility test, the developed film also showed inhibitory effects on all filamentous fungi tested (Table 2). The most significant effect was observed on *Rhizopus stolonifer* and *Microsporum fulvum*. A study by Gopalakrishnan *et al*., 1997 showed high inhibitory activities of α-mangostin-derivatives against three phytopathogenic fungi, namely *Fusarium oxysporum vasinfectum*, *Alternaria tenuis* and *Drechslera oryzae*.

Table 2: The antifungal susceptibility test of the developed films on agar diffusion test. The film showed antifungal activity on all test fungi.

Test fungi	Antimicrobial Activity	
	Pericarp extract loaded film	Negative control
A. niger	+	-
R. stolonifera	+++	-
M. fulvum	+++	-
F. solani	++	-

-: No inhibition, +: Diameter of inhibition zone >15 mm, ++: Diameter of inhibition zone >20mm, +++: Diameter of inhibition zone>25mm

In Hohenstain challenge test, all test microorganisms showed significant growth reduction with the treatment of the *G. mangostana* extracts loaded film. In general, 3 out of 6 test microorganisms showed 99% of growth reduction relative to control. The results obtained are in agreement with previous study where α-mangostin exhibited significant inhibitory activity on *S. aureus*, *P. aeruginosa*, *Salmonella typhimurium* and *Bacillus subtilis* (Gopalakrishnan *et al*., 1997). Due to the control release property of the film, no regrowth of microorganisms was observed throughout the test period on 4 test bacteria, even the films were washed 10 times with detergent (Table 3). These results revealed that the binding of the *G. mangostana* extracts to the nano-cellulose is strong and thus enable the film to withstand the effect of washing. In addition, the excellent binding mechanisms from gelatin also increased the laundering durability. However, the antimicrobial efficiency was significantly reduced after 20 rounds of launderings.

Table 3: The antimicrobial efficiency of the developed film on Hohenstein challenge test. All the test microorganisms showed significant growth reduction with the treatment of *G. mangostana* L extract loaded film.

Test bacteria	Percentage of growth reduction (%)		
	0 wash	10 washes	20 washes
MRSA	95.6	86	70.5
Streptococcus sp.	99.9	90.9	85.5
B. cereus	99.8	90.9	86.6
E. coli	90.5	85.5	60.9
Yersinia sp.	97.5	90.6	86.6
P. aeruginosa	99.9	89.9	75.6

Since *G. mangostana* extracts contain high concentration of α-mangostin, and hence this compound was selected as a reference compound in the *in vitro* release study of the developed film. The release of α-mangostin from the developed nano-cellulose film was evaluated at skin temperature for duration of 72 h (Figure 2). No burst release effect was detected during the test period (72 h). The α-mangostin release reaches plateau conditions at 48 h with a total release of 23.5 µg/mL of α-mangostin from the film. The *in vitro* release of the α-mangostin shows that the developed films provide a constant drug release into the wound without a sudden or rapid increase of drug concentration. Such release pattern suits the application of the film as wound dressing for the wound management of foot ulcer whereby the steady release of the antimicrobial agent will ensure long term antimicrobial effect of the film. Moreover, the gelatin in the nano-cellulose serves as a diffusion barrier which prevents the burst release of the extract. The combination of crystalline cellulose from cotton and reinforcing gelatine film is an ideal material for wound dressing. It is noteworthy that the film prepared with the *G. mangostana* extracts showed significant antimicrobial properties against the infection causing bacteria.

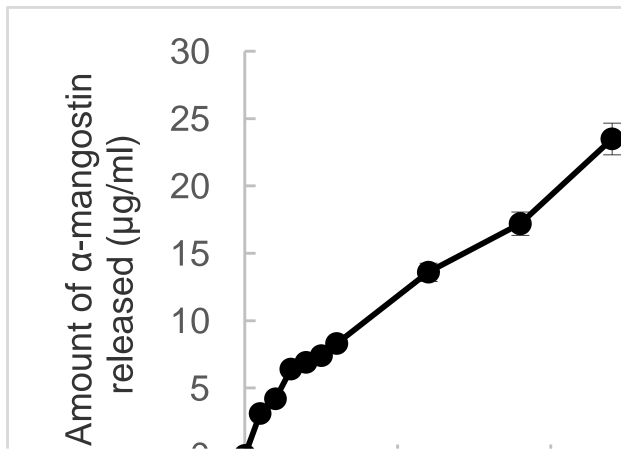

Figure 2: The amount of α-mangostin released from the nano-cellulose film loaded with *G. mangostana* L pericarps extract. No burst release effect was observed.

CONCLUSION

In conclusion, the *G. mangostana* L pericarp extract loaded nano-cellulose films exhibited significant inhibitory activity on diabetic wound microorganisms. The developed film can be potentially used to prevent foot ulcer infection among diabetic patients.

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