Pharmaceutical nanotechnology

Electrospun chitosan-based nanofiber mats loaded with Garcinia mangostana extracts

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A B S T R A C T

The aim of this study was to prepare electrospun chitosan-based nanofiber mats and to incorporate the fruit hull of Garcinia mangostana (GM) extracts into the mats. Chitosan-ethylenediaminetetraacetic acid/polyvinyl alcohol (CS-EDTA/PVA) was selected as the polymers. The GM extracts with 1, 2 and 3 wt% α-mangostin were incorporated into the CS-EDTA/PVA solution and electrospun to obtain nanofibers. The morphology and diameters of the mats were analyzed using scanning electron microscopy (SEM). The mechanical and swelling properties were investigated. The amount of GM extracts was determined using high-performance liquid chromatography (HPLC). The antioxidant activity, antibacterial activity, extract release and stability of the mats were evaluated. In vivo wound healing tests were also performed in Wistar rats. The results indicated that the diameters of the fibers were on the nanoscale and that no crystals of the extract were observed in the mats at any concentration. The mats provided suitable tensile strength and swelling properties. All of the mats exhibited antioxidant and antibacterial activity. During the wound healing test, the mats accelerated the rate of healing when compared to the control (gauze-covered). The mats maintained 90% of their content of α-mangostin for 3 months. In conclusion, the chitosan-based nanofiber mats loaded with GM extracts were successfully prepared using the electrospinning method. These nanofiber mats loaded with GM extracts may provide a good alternative for accelerating wound healing.

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1. Introduction

Chitosan is a copolymer of N-acetyl-D-glucosamine (Glc-NAC) and D-glucosamine (GlcN) that is produced by alkaline deacetylation of chitin. Chitosan is biodegradable, biocompatible, and non-toxic; therefore, it has been used as a material for use in biomedical applications (Dash et al., 2011; Rinaudo, 2006). Chitosan has been considered as a wound dressing material due to the special properties as proliferation, antioxidant, antibacterial, activates macrophages and hemostasis. Moreover, chitosan will gradually depolymerize into N-acetyl-D-glucosamine, which initiates fibroblast proliferation, assists in ordered collagen deposition and stimulates increased levels of natural hyaluronic acid synthesis at the wound site. Because of these reasons, chitosan have been one of the important biomaterials for wound management in recent years (Jayakumar et al., 2011; Muzzarelli, 2009; Paul and Sharma, 2004). Hence chitosan biopolymer is the one choice for developing nanofibrous wound dressing material via electrospinning technique. The electrospun nanofiber is appropriate for use as a wound dressing material due to its useful properties, including oxygen-permeable high porosity, variable pore-size distribution, high surface to volume ratio, and most importantly, morphological similarity to the natural extracellular matrix (ECM) in the skin, all of which promote cell adhesion, migration and proliferation (Jayakumar et al., 2011; Zahedi et al., 2010). For wound dressing applications, the electrospun nanofiber can be used with or without agents that promote wound healing and the polymer must be biocompatible, biodegradable and low toxicity (Heunis and Dicks, 2010; Venugopal and Ramakrishna, 2005).

Recently, chitosan nanofibers have been successfully generated from the electrospinning of pure chitosan, chitosan derivatives and chitosan blends with other polymers. For wound healing applications, blended chitosan was used to prepare nanofiber such as chitosan/collagen (J.-P. Chen et al., 2008; Wang et al., 2008), chitosan/silk (Cai et al., 2010), carboxyethyl chitosan/polyvinyl alcohol (PVA) (Zhou et al., 2008) and quaternary chitosan/polyvinyl pyrrolidone (PVP) (Ignatova et al., 2007). However, the chitosan electrospun nanofiber which incorporated wound healing enhancement agent has been few reported for wound dressing application. Many varieties of active agent have been used to accelerate wound healing process such as antimicrobials and antibiotic, epithelia growth factor, fibroblasts growth factor, vitamin and...
mineral, nanosilver particles and agent of high potential in a wound dressing material from natural extracts (Zahedi et al., 2010). Mangosteen (Garcinia mangostana Linn.; GM) is a tropical fruit found in Southeast Asia. People in Southeast Asia have used the pericarp (peel, rind, hull or ripe) of GM as a traditional medicine for the treatment of abdominal pain, diarrhea, dysentry, infected wound, suppuration, and chronic ulcer. The pericarp of GM was reported to be a good source of xanthone, α-, β-, and γ-mangostins, garcinone E, 8-deoxygartanin, and gartanin (Pedraza-Chaverri et al., 2008). Several studies have revealed that GM extracts exhibit antimicrobial (Chommawang et al., 2009; Pothitirat et al., 2009a; Sundaram et al., 1983), antiroliferative (Moongkarndi et al., 2004), antioxidant (Palakawong et al., 2010; Weecharangsan et al., 2006; Williams et al., 1995), anti-inflammatory (L.-G. Chen et al., 2008) and analgesic (Cui et al., 2010). Because of these useful activities of GM extracts, it can be used for wound healing application.

In our previous study, the GM extracts were successfully loaded in PVA electrospun nanofiber mats which still remain the antioxidant activity for cosmetic applications (Opanasopit et al., 2008). Chitosan was also successfully prepared in aqueous salt to form nanofibers without the use of organic solvents or toxic acids such as chitosan-hydroxybenzotriazole (CS-HOBr)/PVA (Charernsriwilaiwat et al., 2010), chitosan-thiamine pyrophosphate (CS-TPP)/PVA (Charernsriwilaiwat et al., 2012c) and chitosan-ethylenediaminetetraacetic acid (CS-EDTA)/PVA (Charernsriwilaiwat et al., 2011). Among the obtained fibers, CS-EDTA/PVA nanofiber mats showed the excellent antibacterial, antioxidant activity and performed better than gauze in decreasing acute wound size during the first week after tissue damage (Charernsriwilaiwat et al., 2012b). Moreover, in the commercial product, EDTA was also used for irreversible deactivation of matrix metalloproteinases (MMPs) which play an important role in damaging the extracellular matrix and the extracellular growth factors present in a chronic wound (Fitzgerald and Steinberg, 2009).

The aim of this study was to prepare chitosan-based nanofiber mats loaded with GM extracts to enhance their efficacy as antibacterial and antioxidant agents for enhancing wound healing. The morphology and structure of the chitosan-based nanofiber mats after the extract was loaded were analyzed using scanning electron microscopy (SEM). The composites and the thermal behaviors of the nanofiber mats were characterized using Fourier transform infrared spectrophotometer (FT-IR) and differential scanning calorimeter (DSC). The mechanical and swelling properties of the mats were investigated. α-Mangostin (see the chemical structure, Fig. 1) was used as a marker of the GM extract. The amount of GM extracts (α-mangostin) remaining in the chitosan-based nanofiber mats was determined using high-performance liquid chromatography (HPLC). The release profile of α-mangostin from the nanofiber mats loaded with GM extracts was analyzed using Franz’s diffusion cell. The cytotoxicity tests for the nanofiber mats were evaluated with a MTT assay using human fibroblast cells. The antioxidant and antibacterial activities of the nanofiber mats were analyzed. The in vivo wound healing effects of the nanofiber mats were investigated using an animal model. The stability of the nanofiber mats was studied by storing the mats under normal conditions and comparing them with mats stored under stress conditions.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation 0.85, MW 110 kDa) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma–Aldrich Chemical Company, USA. Polyvinyl alcohol (PVA) (degree of polymerization ≈ 1600, degree of hydrolysis ≈ 97.5–99.5 mol%) was purchased from Fluka, Switzerland. G. mangostana (GM) was obtained from a farm in Chantaburi Province, Thailand. Normal human foreskin fibroblast (NHF) cells were obtained from the American Type Culture Collection (ATCC) in Rockville, MD, USA. Dimethyl sulfoxide (DMSO) was obtained from BDH Laboratories, UK. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Trypsin–EDTA, and penicillin–streptomycin were purchased from Gibco BRL, Rockville, MD, USA. All other reagents and solvents were commercially available and were of analytical grade.

2.2. Preparation of GM extracts

The hulls of GM were cut into small pieces and dried in a hot air oven at 50 °C for 24 h. The dried samples were milled into powder by blender. Dried powder was separately macerated with 70% acetone at room temperature until the extraction was exhausted. Acetone extract was combined and filtered through a Whatman no. 1 filter paper under suction. The filtrate was concentrated on water bath and evaporates solvent in rotary evaporator to obtain the dry crude extracts.

2.3. Standardization of GM extracts

2.3.1. α-Mangostin content in GM extracts

The GM extracts were analyzed by the amount of α-mangostin determined by HPLC (Agilent Technology, USA). A VertiSep® AQS C18 column (250 mm × 4.6 mm, 5 μm particle size) with a C18 guard column was used. The HPLC analysis was performed according to the method of Pothitirat et al. (2009a) with a slight modification. The elution was performed using gradient solvent systems that consisted of acetonitrile (mobile A) and 0.1% (v/v) ortho phosphoric acid (mobile B) with a flow rate of 1 ml/min at ambient temperature. The gradient program was as follows: 70% A for 0–15 min, 70–75% A in 3 min, 75–80% A in 1 min, constant at 80% A for 6 min, and 80–70% A in 1 min. The wavelength of the UV-visible detector was set at 320 nm. The content of α-mangostin was calculated using its calibration curve with respect to the dilution factor and was expressed as gram per 100 g of the extract.

2.3.2. Antioxidant activity of the GM extracts

2.3.2.1. Free radical scavenging activity for 2,2-diphenyl-1-picrylhydrazyl (DPPH). A 200 μM aliquot of DPPH in methanol (100 μl) was added to 100 μl of the GM extract. The extracts were dissolved by their solvent and then diluted to the desired concentration with methanol. The mixture was held at room temperature for 30 min. The absorbance was measured at 550 nm (Fusion Universal Microplate Analyser Model: A153601, Perkin Elmer Life and Analytical Sciences, Inc., USA). The results of the assay were expressed as IC50, which represents the concentration...
of the extract (µg/ml) required to inhibit 50% of the free radical scavenging activity. The free radical scavenging activity was assessed using Eq. (1):

\[
\text{\% Inhibition} = \left( 1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]  

(1)

where \(A_{\text{sample}}\) is the absorbance in the presence of the extracts and \(A_{\text{control}}\) is the absorbance of the control. The \(IC_{50}\) values were calculated by linear regression of the plots where the x-axis represented the various concentrations (µg/ml) of the GM extracts and the y-axis represented the % inhibition.

2.3.2.2. Scavenging activity for 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals. The ABTS working solution was prepared by reacting an ABTS solution (7 mM) with potassium persulfate solution (4.95 mM). The mixture solution was reacted in the dark at room temperature for 12–16 h before use. Before the assay, the solution was diluted in a phosphate buffer at a pH of 7 to yield an absorbance of 0.7 ± 0.02 at 734 nm. Then, 3.9 ml of the working solution was mixed with 0.1 ml of the sample. After 10 min at room temperature, the absorbance at 734 nm was measured. The percent inhibition was then calculated using Eq. (1), and the \(IC_{50}\) value was calculated following the procedure described for the DPPH assay.

2.3.2.3. Ferric reducing/antioxidant potential (FRAP) assay. The FRAP solution was freshly prepared by mixing an acetate buffer at a pH of 3.6 (a), 20 mM of a ferric chloride solution (b) and 10 mM of a tripyridyl-s-triazine (TPTZ) solution (c) in a 10:1:1 (a:b:c) ratio. The sample solution (50 µl) was added to the FRAP reagent (950 µl). The mixture was incubated for 30 min at room temperature, and the absorbance was measured at 593 nm. The measurement was compared to a standard curve for FeSO₄·H₂O solutions and expressed as an \(EC_{1}\) value, which indicates that the concentration of antioxidant in the reactive system has a ferric–TPTZ reducing ability equivalent to that of 1 mM of FeSO₄·H₂O.

2.3.2.4. Metal ion chelating assay. The ferrous ion–chelating potential of the GM extract was investigated according to the method of Decker and Welch (1990). The ferrous-ion–chelating ability was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, the reaction mixture was composed of varying concentrations of the GM extract, FeCl₂ (2 mM) and ferrozine (5 mM) and adjusted to a total volume of 0.8 ml with water, shaken well and incubated for 10 min at room temperature. The absorbance was then measured at 562 nm. The percent chelating activity was then calculated following Eq. (1), and the \(IC_{50}\) value was calculated following the procedure described for the DPPH assay.

2.3.3. Determination of total phenolic content

To ensure that every batch of the extract has uniformity and consistency for the exhibited biological effects, the total phenolic and tannic acid content and the extraction yield were determined. The amounts of phenolic compounds in the extracts were determined using the Folin–Ciocalteu method, which was adapted from Singleton et al. (1999), and gallic acid was used as the standard phenolic compound. Fifty-microliter aliquots of the extracts (1 mg/ml) were added to a mixture of 2.5 ml of 10% Folin–Ciocalteu reagent and 2 ml of 7.5% Na₂CO₃. After incubation at 45 °C for 30 min, the absorbance was measured at 765 nm. A linear dose–response regression curve was generated using the absorbance reading of gallic acid. The content of total phenolic compounds in the extract was expressed as grams of gallic acid equivalent per 100 gram of dry weight (g GAE/100 g) of extracts.

2.3.4. Determination of total tannins content

The total tannins content in the extracts was determined using the method that was adapted from Silber and Fellman (2006). First, 2 mg of bovine serum albumin was mixed with 1 ml of the sample extract at a concentration of 1 mg/ml and then maintained at room temperature for 20 min. Then, the mixtures were centrifuged, and the sediment was dissolved with 0.1% sodium dodecyl sulfate (2 ml), triethanolamine (2 ml) and 10 mM FeCl₃ (1 ml). The absorbances of the suspensions were measured at 510 nm. The calibration curve was established using standard tannic acid. The content of total tannin in the extract was expressed as grams of tannic acid equivalent per gram of dry weight (g TAE/100 g) of extracts.

2.4. Preparations of GM extracts loaded chitosan-based nanofiber mats

The 2% (w/v) chitosan (CS) solution was prepared by dissolving CS and EDTA in distilled water at a weight ratio of 2:1. The 10% (w/v) PVA solution was prepared by dissolving PVA in distilled water at 80 °C, followed by stirring for 4 h. The CS-EDTA solution was mixed with a PVA solution at a weight ratio of 30/70. GM extracts (containing 1, 2 and 3 wt% α-mangostin to polymer) were added into the 30/70 CS-EDTA/PVA solution, and then, the solution was stirred for 24 h. The viscosity, conductivity and surface tension of the solutions were measured. The electrospinning solution was contained in a 5 ml glass syringe connected with a 20-gauge stainless steel needle (diameter = 0.9 mm) at the nozzle. The needle was connected to the positive polarity emitting electrode of a Gamma High Voltage Research device. The electric potential was fixed at 15 kV. The nanofibers were collected as-spun on an aluminum sheet that was wrapped on a rotating collector. The solution feed was driven by a syringe pump, and the rate was fixed at 0.25 ml/h during spinning. The solution was electrospun at room temperature, and the collection distance was fixed at approximately 20 cm. The process duration was fixed at 24 h to provide mats with a 20–30 µm thickness.

2.5. Characterizations of nanofiber mats

The morphology and diameter of the nanofiber mats were determined using scanning electron microscopy (SEM, Camscan Mx2000, England). For this process, a small section of the nanofiber mats was sputtered with a thin layer of gold before the SEM observations. The average diameter and diameter distribution of the nanofiber mats were analyzed by randomly measuring the diameters of the nanofibers at 100 different points from the SEM images using image analysis software (JMicroVision V.1.2.7, Switzerland).

The chemical structure of the nanofiber mats was characterized using a Fourier transform infrared spectrophotometer (FT-IR, Nicolet 4700, Becthai, USA) with a wave number range of 400–4000 cm⁻¹. The thermal behavior of the nanofiber mats was evaluated by differential scanning calorimeter (DSC, Pyris Sapphire DSC, PerkinElmer Instrument, USA) under an atmosphere of nitrogen. DSC traces were recorded from 25 to 250 °C at a heating rate of 5 °C/min.

The mechanical properties of the nanofiber mats were evaluated using a texture analyzer (TA.XT plus, Stable Micro Systems, UK) with a 5 kg load cell equipped with a tensile grips holder. The samples were cut into a rectangular shape (5–25 mm). The thicknesses of these samples ranged from 20 to 30 µm.
The swelling degree of the nanofiber mats were investigated in a phosphate buffer solution at a pH of 7.4 at room temperature for 1 h according to Eq. (2):

\[
\text{Degree of swelling (\%)} = \frac{M - M_d}{M_d} \times 100
\]  

(2)

where \( M \) is the weight of each sample after immersion in the buffer solution for 1 h and \( M_d \) is the initial weight of the sample in its dry state.

The loading efficacy of the GM extracts into the CS-EDTA/PVA nanofiber mats was determined by submerging the mats (5 mg) into 5 ml of an acetate buffer (pH 5.5) and methanol (50:50) for 24 h. Then, 1 ml of the solution was analyzed using HPLC to determine the amount of \( \alpha \)-mangostin, which is used as a marker in the nanofiber mats, as mentioned in Section 2.3.1. The amount of \( \alpha \)-mangostin was used to calculate the amount of GM extracts in the nanofiber mats. The % loading efficacy was calculated using Eq. (3):

\[
\text{Loading efficacy (\%)} = \left( \frac{L_a}{L_t} \right) \times 100
\]  

(3)

where \( L_a \) is the amount of the GM extracts that are embedded in the nanofibers and \( L_t \) is the theoretical amount of GM extracts (obtained from the feeding condition) incorporated into the nanofibers.

The antioxidant activity of the CS-EDTA/PVA nanofiber mats loaded with the GM extracts was investigated as described in Section 2.3.

2.6. In vitro release study

The release characteristics of the CS-EDTA/PVA nanofiber mats loaded with GM extracts were investigated using Franz’s diffusion cells with a water jacket connected to a water bath at 37 °C; each cell had a volume of 6.5 ml and an effective diffusion area of 2.43 cm². The receiver compartments were filled with an acetate buffer (pH 5.5) and methanol (50:50) and stirred with a Teflon magnetic stirrer at 600 rpm. The nanofiber mats were cut with an equal diameter effective diffusion area and were mounted between two half cells of the diffusion cells. At a given time interval, an aliquot (1.0 ml) of the receiver solution was withdrawn and replaced with the same volume of fresh medium to maintain a constant volume. The amount of GM extracts in the sample solutions was analyzed by HPLC. The obtained data were carefully analyzed to determine the cumulative amount of GM extracts released from the specimens at each immersion time point. The experiments were conducted in triplicate.

2.7. Indirect cytotoxicity

The cytotoxicity of the nanofiber mats was evaluated based on a procedure adapted from the ISO10993-5 standard test method (indirect contact). The nanofiber mats were sterilized with UV radiation for 1 h. The mats were then immersed in a serum-free medium (SFM; containing Dulbecco’s modified Eagle’s medium (DMEM), 1% (v/v) t-glutamine, 1% (v/v) lactalbumin and 1% (v/v) antibiotic and anticycnic formulation) in an incubator for 24 h to produce different concentrations of extraction media (1, 2.5, 5, 7.5 and 10 mg/ml). Normal human foreskin fibroblast (NHF) cells were plated in 100 μl of DMEM, which was supplemented with 10% FBS, at a density of 8000 cells/well in 96-well plates. When the cultures reached confluency (typically 48 h after plating), the varying concentrations of the tested extraction media were replaced, and the cells were re-incubated for 24 h. After treatment, the tested extraction solutions were removed. Finally, the cells were incubated with 100 μl of a MTT-containing medium (1 mg/ml) for 4 h. The medium was removed, the cells were rinsed with PBS (pH 7.4), and the formazan crystals that formed in living cells were dissolved in 100 μl of dimethylsulfoxide per well. The relative cell viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model A0PUS01 and A153601, Packard BioScience, CT, USA). The viability of the non-treated control cells was defined as 100%.

2.8. Antibacterial activity

The antibacterial activity of the nanofiber mats was tested against Staphylococcus aureus (S. aureus) ATCC 6538P and Escherichia coli (E. coli) ATCC 10536. For the minimum inhibitory concentration (MIC) test, S. aureus and E. coli were cultivated in Tryptone soy broth (TSB) in a shaking incubator at 37 °C and 100 rpm for 24 h. The bacterial suspension was diluted until the bacterial concentration was approximately \( 1 \times 10^8 \) cfu/ml and it was then pipetted into a 24 well plate at a concentration of 1 ml/well. The different weights of the nanofiber mats (0.25–5 mg) were placed into the wells that contained the bacterial suspension and incubated at 37 °C for 24 h. The MIC was defined as the minimum concentration of mats where no growth was observed after 24 h of incubation. The optical density (OD) at 550 nm was measured using a microplate reader. For determining the minimum bactericidal concentration (MBC), the mixtures from the wells with no growth (100 μl) were spread onto agar plates for the MBC determination. The MBC was defined as the minimum concentration of mats where no colony growth was observed on the agar plates after 24 h of incubation at 37 °C. The MIC and MBC determinations were conducted in triplicates, and 1 mg/ml penicillin was used as a positive control.

2.9. Wound healing test

This study was approved by an Investigational Review Board (Animal Studies Ethics Committee, Faculty of Pharmacy, Silpakorn University, Approval No. 2-2553). Male Wistar rats (240–280 g) were used in this study. After anesthetization, the neck area of the dorsal of each rat was shaved and wiped with 70% ethanol. Two wounds were created on the neck area of each rat using a skin biopsy punch (wound area of 0.8 cm²). The wound was treated by placing an equal size of nanofiber mat, gauze and commercial antibacterial gauze dressing (Sofra-tulle®, Sanofi Aventis, UK) (n=6) over it without removing the test material throughout the study period. The area of the wound was measured daily using the planimetry method until the wound completely healed. The percentage of wound closure is defined as Eq. (4):

\[
\text{Wound closure (\%)} = \left( 1 - \frac{A_i}{A} \right) \times 100
\]  

(4)

where \( A_i \) is the initial wound area and \( A \) is the wound area after a fixed time interval.

2.10. Histological examination

The rats were sacrificed at day 11 after operation. The trauma samples were cut and fixed in 10% neutral buffered formalin, embedded in paraffin, and serially sectioned at 5 μm. The nuclei of the sections were stained with hematoxylin, rinsed in running tap water, differentiated with 0.3% acid alcohol, rinsed in running tap water, stained with eosin for 2 min, then dehydrated, cleared and mounted. The hematoxylin–eosin stained sections were observed under a light microscope (Nikon Inverted-Eclipse TE2000-U, Japan).

2.11. Stability studies

The stability of the CS-EDTA/PVA nanofiber mats loaded with GM extracts was monitored up to 6 months under stress conditions.
Table 1

<table>
<thead>
<tr>
<th>Test</th>
<th>GM extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolics (g GAE/100 g of extract)</td>
<td>26.46 ± 0.22</td>
</tr>
<tr>
<td>Total tannins (g TAE/100 g of extract)</td>
<td>34.05 ± 0.05</td>
</tr>
<tr>
<td>α-Mangostin (% (w/v) of extract)</td>
<td>13.20 ± 0.20</td>
</tr>
<tr>
<td>Antioxidant activities</td>
<td></td>
</tr>
<tr>
<td>DPPH IC50 (µg/ml)</td>
<td>14.65 ± 0.16</td>
</tr>
<tr>
<td>ABTS IC50 (µg/ml)</td>
<td>1.67 ± 0.03</td>
</tr>
<tr>
<td>FRAP EC1 (ng/ml)</td>
<td>0.33 ± 0.001</td>
</tr>
<tr>
<td>Metal ion chelating IC50 (µg/ml)</td>
<td>No</td>
</tr>
</tbody>
</table>

(45 °C 75%RH) and compared to normal conditions (25 °C 40%RH). The content of α-mangostin, the antioxidant activity (DPPH assay) and tensile strength of the nanofiber mats were determined every 1 month. The physical appearance and morphology of the nanofiber mats were investigated using SEM every 3 months. The % of GM extracts and the tensile strength remaining in the mats was calculated as Eq. (5).

Remaining GM extracts or tensile strength (%) = \( \left( \frac{V_f}{V} \times 100 \right) \)  

where \( V_f \) is the initial amount of GM extracts or tensile strength and \( V \) is the amount of GM extracts or tensile strength after a fixed sampling time.

2.12. Statistical analysis

All experiment data were collected from triplicate samples and are expressed as the mean ± standard deviation (S.D.). Statistically significant differences in cell viability, wound area and stability study were examined using the Student’s t-test. The significance level was set at \( p < 0.05 \).

3. Results and discussion

3.1. Standardizations of GM extracts

The GM extracts were standardized before being loaded into the CS-EDTA/PVA solution. The total phenolics, total tannins, and α-mangostin content in the GM extracts and the antioxidant activity are shown in Table 1. The total phenolics and total tannins were 26.46 ± 0.22 g GAE/100 g of extract and 34.05 ± 0.05 g TAE/100 g of extract, respectively. The content of α-mangostin in the GM extracts was 13.20 ± 0.20% (w/v). The antioxidant activities of the GM extracts solution were determined with DPPH, ABTS, FRAP and metal ion chelating. The IC50 values for the DPPH and ABTS assay were 14.66 ± 0.16 µg/ml and 1.67 ± 0.03 µg/ml, respectively. The EC1 value for the FRAP assay was 0.33 ± 0.001 ng/ml. For metal ion chelating, the GM extracts did not exhibit the chelating ability. These results were in agreement with Pathitirat et al. (2009a), who reported that the total phenolics and total tannins of a 95% (v/v) ethanol solution containing GM extracts were 28.88 ± 0.73 g GAE/100 g of extract and 36.66 ± 0.43 g GAE/100 g of extract, respectively. The α-mangostin content in a 95% (v/v) ethanol solution containing GM extracts was 13.63 ± 0.06% (w/w) (Pathitirat et al., 2009a). These results confirm that the 70% (v/v) acetone extractions still contained α-mangostin, phenolic compounds, tannins and antioxidant properties.

3.2. Electrospinning

The CS-EDTA/PVA (30/70) solution containing various amount of GM extracts (containing 0, 1, 2 and 3 wt% α-mangostin to polymer) was prepared. The viscosity, conductivity and surface tension of the solutions were measured and are shown in Table 2. When the amount of GM extracts was greater than 3 wt% α-mangostin, the properties of the solution were degraded, such as very low viscosity, and the fiber mats could not form. There were many beads in the structure, and it became a droplet on the collector. Fig. 2 shows the SEM image and the diameter distributions of the CS-EDTA/PVA nanofiber mats with and without various amounts of GM extracts. The SEM images revealed that all of the fibers were smooth, and did not contain the crystals of GM extracts in their structure. This result indicated that the GM extracts were well incorporated within the fibers. The diameters of the fiber mats loaded with GM extracts were in the nanometer range. The diameter distributions of these fibers are shown in Fig. 2. The diameter of the nanofiber and the % loading efficacy of the GM extracts in the nanofiber mats slightly increased from 205.56 to 251.35 nm and from 40.69 to 43.86%, respectively, when the amounts of GM extracts were increased (Table 3). This result indicates the limited incorporation of GM extracts into the nanofiber mats. The solution parameters of the CS-EDTA/PVA solution loaded with GM extracts were slightly altered and did not affect the morphology of the fibers (Table 2). This result was in accordance with our previous study, which indicated that the incorporation of GM extracts into the electrospun PVA did not affect their morphology because the surface of the PVA fibers loaded with GM extracts was also smooth, and the average diameters of both the bare and the PVA fibers loaded with GM extracts ranged between 140.7 and 197.3 nm (Opasanopit et al., 2008). In addition, loading the CS-EDTA/PVA fibers with lysozymes did not affect their morphology, and the average diameters were in the range of 143–209 nm (Charernsriwilaiwat et al., 2012a).

3.3. Characterizations

The FT-IR spectra of the CS-EDTA/PVA nanofiber mats with and without different amount of GM extracts and α-mangostin powder are shown in Fig. 3a. The spectrum of the 0 wt% α-mangostin fiber mats exhibited absorption peaks at 3360, 2940, 1650, 1430 and

Table 2

<table>
<thead>
<tr>
<th>CS-EDTA/PVA solution</th>
<th>Viscosity (mPa s)</th>
<th>Conductivity (µS cm⁻¹)</th>
<th>Surface tension (mN m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% α-mangostin</td>
<td>254.7 ± 0.76</td>
<td>1006.0 ± 7.55</td>
<td>55.5 ± 0.28</td>
</tr>
<tr>
<td>1% α-mangostin</td>
<td>252.4 ± 2.39</td>
<td>1030.5 ± 2.65</td>
<td>53.9 ± 0.57</td>
</tr>
<tr>
<td>2% α-mangostin</td>
<td>245.9 ± 1.37</td>
<td>1075.7 ± 1.53</td>
<td>52.1 ± 0.31</td>
</tr>
<tr>
<td>3% α-mangostin</td>
<td>239.9 ± 1.64</td>
<td>1114.7 ± 8.74</td>
<td>49.3 ± 0.15</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>GM extracts</th>
<th>Fiber diameter (nm)</th>
<th>Loading efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% α-mangostin</td>
<td>205.5 ± 36.06</td>
<td>-</td>
</tr>
<tr>
<td>1% α-mangostin</td>
<td>207.8 ± 34.30</td>
<td>40.69 ± 2.30</td>
</tr>
<tr>
<td>2% α-mangostin</td>
<td>202.7 ± 36.74</td>
<td>41.97 ± 3.25</td>
</tr>
<tr>
<td>3% α-mangostin</td>
<td>251.3 ± 47.95</td>
<td>43.86 ± 0.11</td>
</tr>
</tbody>
</table>
1095 cm\(^{-1}\), which were attributed to \(\nu(O-H)\), \(\nu_s(CH)\), \(\nu(C=O)\), \(\delta(CH-O-H)\) and \(\nu(C-O)\), respectively (Charernsriwilaiwat et al., 2011). In the spectrum for the pure \(\alpha\)-mangostin powder, the dominant absorption peaks were observed at 3422, 1642 and 1284 cm\(^{-1}\), which correspond to the phenolic, carbonyl and methoxy groups, respectively (Ghazali et al., 2010). The peak that was observed for the \(\alpha\)-mangostin powder was also observed in spectra of the 1–3 wt% \(\alpha\)-mangostin loaded nanofiber mats. The results indicated that \(\alpha\)-mangostin in the GM extracts was well incorporated into the nanofiber mats.

Fig. 3b shows the DSC thermograms of the CS-EDTA/PVA nanofiber mats with and without different amounts of the GM extracts and the \(\alpha\)-mangostin powder. The endothermic curves of the nanofiber mats indicated that the melting point slightly decreased from approximately 216.9°C to 215.2, 212.4 and 210.2°C when the amount of GM extracts increased to 1, 2 and 3 wt% \(\alpha\)-mangostin, respectively. For the pure \(\alpha\)-mangostin powder, the melting point was observed at approximately 183.7°C, which is lower than that of the nanofiber mats. This result indicates that the amount of GM extracts in the fiber mats does not affect their thermal behavior.

The mechanical properties of the wound dressing are important when interacting with the wound; it must possess properties similar to those of skin to function properly until the wound is healed. The mechanical properties in terms of the tensile strength, strain at the maximum and the Young's modulus of the CS-EDTA/PVA nanofiber mats with and without different amounts of GM extracts were characterized, and the results are presented in Table 4. The tensile strength of all of the tested nanofibers mats was in the range of 4.57–5.11 MPa, and the average value was 4.76 MPa. When the amount of the GM extracts increased, the tensile strength of the fiber mats slightly decreased. The strain at the maximum of all of the tested nanofibers mats was in the range of 7.06–7.61 MPa, and the average value was 7.34 MPa. The Young's modulus of all of the tested nanofibers mats was in the range of 51.9–87.8 MPa, and the average value was 67.7 MPa. The results indicated that the GM extracts in the fiber mats exerted less effects on the mechanical properties of the mats. These findings illustrated that these CS-EDTA/PVA fiber mats loaded with GM extracts have the potential to be developed as a wound dressing.

The swelling of the fiber mats is the most important property that characterizes its use for wound dressing applications. Fig. 4 shows the degree of swelling of the CS-EDTA/PVA nanofiber mats with and without different amounts of GM extracts after immersion in a phosphate buffer solution (pH 7.4) at room temperature for 1 h. The bare nanofiber mats (0 wt% \(\alpha\)-mangostin to polymer) demonstrated a swelling degree of 134.53%, which slightly decreased with increasing amounts of the GM extracts. The swelling degree of the GM extracts (1, 2 and 3 wt% \(\alpha\)-mangostin to polymer) loaded
Table 4
Mechanical properties of the GM extracts (0, 1, 2 and 3 wt% α-mangostin to polymer) loaded CS-EDTA/PVA nanofiber mats. Each value represents the mean ± S.D. from three independent experiments.

<table>
<thead>
<tr>
<th>CS-EDTA/PVA fiber mats</th>
<th>Tensile strength (MPa)</th>
<th>Strain at maximum (%)</th>
<th>Young’s modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% α-mangostin</td>
<td>5.11 ± 0.33</td>
<td>7.06 ± 3.06</td>
<td>60.8 ± 16.0</td>
</tr>
<tr>
<td>1% α-mangostin</td>
<td>4.77 ± 0.56</td>
<td>7.33 ± 2.97</td>
<td>51.9 ± 0.9</td>
</tr>
<tr>
<td>2% α-mangostin</td>
<td>4.59 ± 0.16</td>
<td>7.61 ± 0.45</td>
<td>70.4 ± 36.7</td>
</tr>
<tr>
<td>3% α-mangostin</td>
<td>4.57 ± 0.17</td>
<td>7.36 ± 0.55</td>
<td>87.8 ± 34.2</td>
</tr>
</tbody>
</table>

Fig. 4. Degree of swelling (%) of the GM extracts loaded CS-EDTA/PVA nanofiber mats with different amount of GM extract (0, 1, 2 and 3 wt% α-mangostin to polymer). The data are expressed as mean ± S.D. from three independent experiments.

Fiber mats were 111.96, 101.7 and 96.67%, respectively. Chitosan is a hydrophilic polymer, and water diffuses very rapidly through this material before its degradation (Baskar and Kumar, 2009). The GM extract is a hydrophobic compound; therefore, it might affect the swelling properties of the chitosan-based nanofiber mats by increasing the hydrophobicity of the mats, which caused the decrease in the swelling degree.

3.4. In vitro study

The release profile of α-mangostin from the CS-EDTA/PVA nanofiber mats loaded with GM extracts was investigated using Franz’s diffusion cells. Fig. 5 shows the α-mangostin release characteristics from the nanofiber mats with different amounts of GM extracts (containing 1, 2 and 3 wt% α-mangostin to polymer).

Fig. 5. α-Mangostin release profiles from the GM extracts loaded CS-EDTA/PVA nanofiber mats with different amounts of GM extract: (●) 1 wt% α-mangostin, (▲) 2 wt% α-mangostin and (■) 3 wt% α-mangostin. The data are expressed as mean ± standard deviation from three independent experiments.

3.5. Indirect cytotoxicity

The toxicity of the GM extracts (containing 0, 1, 2 and 3 wt% α-mangostin to polymer) loaded CS-EDTA/PVA nanofiber mats were investigated on NHF for 24 h using a MTT assay. Fig. 6 shows the cell viability of various concentrations of the extract medium from the nanofiber mats loaded with GM extracts. There was a significant decrease in the cell viability when the NHF cells were incubated with higher concentrations (7.5–10.0 mg/ml) of the extraction media of the chitosan-based nanofiber mats containing GM extracts 1, 2 and 3 wt% α-mangostin when compared with the control (p < 0.05). This result might be because the amount of GM extracts was very high and were toxic to fibroblast cells. However, the cell viability was not statistically different from the control in the concentration range of 1–5 mg/ml of all the GM extracts loaded CS-EDTA/PVA nanofiber mats extract medium. The results

Fig. 6. The percentage cell viability in NHF cells at varying concentration of GM extract loaded CS-EDTA/PVA nanofiber mats: (□) 0 wt% α-mangostin, (●) 1 wt% α-mangostin, (▲) 2 wt% α-mangostin and (■) 3 wt% α-mangostin in NHF cells. Each value represents the mean ± standard deviation of five wells. *Statistically significant (p < 0.05).
indicated that GM extracts (1, 2 and 3 wt% α-mangostin) loaded CS-EDTA/PVA nanofiber mats were safe at the concentrations of 1–5 mg/ml of the mats.

3.6. Antibacterial activity

The antibacterial activities of the GM extracts have been reported against both gram-positive and gram-negative bacteria (Palakawong et al., 2010; Sundaram et al., 1983). α-Mangostin in GM extracts also has potent inhibitory effect against methicillin-resistant S. aureus (Chomnawang et al., 2009). The GM extracts (contain 0, 1, 2 and 3 wt% α-mangostin to polymer) loaded CS-EDTA/PVA nanofiber mats were submerged in the bacteria suspension for 24 h. The fiber mats loaded with GM extracts exhibited concentration-dependent antibacterial activity against S. aureus and E. coli. The 1, 2 and 3 wt% α-mangostin GM extracts loaded fiber mats inhibited S. aureus and E. coli growth at 2, 1 and 0.5 mg/ml, respectively. The 0 wt% α-mangostin mats also inhibited bacteria growth at 5 mg/ml. The MIC and MBC of the CS-EDTA/PVA nanofiber mats loaded with GM extracts are shown in Table 5. The results indicated that the strength of the antibacterial activities of the fiber mats loaded with GM extracts were dependent on the amount of the GM extract. The antibacterial activity concentration of these nanofiber mats was less than 5 mg/ml, which is non-toxic to fibroblast cells. This result indicated that the GM extract still retained its antibacterial activity when it was processed during electrospinning. When the mats containing the GM extracts were submerged in the bacteria suspension, the extracts were released from the mats and exhibited an antibacterial effect. Furthermore, the mats were corroded, and chitosan and EDTA were released into the media (El-Sharif and Hussain, 2011). Finally, the antibacterial activity was enhanced by the GM extract, chitosan and EDTA. The antibacterial mechanism of chitosan is generally attributed to the positive charge of the chitosan molecule, which interferes with the negative charge on the surface of the bacteria. Chitosan could interact with the membrane of the bacteria to alter cell permeability (Sudarshan et al., 1992). Chitosan may also have intracellular targets. Chitosan molecules that dissociated in the solution could bind with DNA and inhibit the synthesis of mRNA and proteins (Rabea et al., 2003).

3.7. Wound healing test

In the wound healing study, two wounds with areas of 0.8 cm² were produced on the neck area of the dorsal of each rat. Fig. 7 shows the % wound closure at 1, 4, 7 and 10 days after treatment with gauze (negative control), commercial antibacterial gauze dressing (Sofra-tulle®) (positive control), (g) 0 wt% α-mangostin and (■) 3 wt% α-mangostin GM extracts loaded CS-EDTA/PVA nanofiber mats. The wound closures gradually increased and fully recovered within 11 days for all treatments. At days 1 and 4 after the treatment, the wound healing with the 3 wt% α-mangostin fiber mats dressing was the fastest (p < 0.05); this mats exhibited the highest % wound closure. At the first week after the operation, the treatment with the 3 wt% α-mangostin fiber mats resulted in faster wound healing than that of gauze and the commercial antibacterial gauze dressing treatment. This result might be due to the potential activities, such as antioxidant, anti-inflammatory and antibacterial of the GM extract, especially α-mangostin, in fiber mats, which rapidly released from the fibers to assist in the acceleration of the healing process (Pedraza-Chaverri et al., 2008). The N-acetyl-D-glucosamine and EDTA produced from the degradation of the CS-EDTA/PVA nanofiber mats also enhanced the wound healing rate by promoting fibroblast proliferation (Jayakumar et al., 2011; Paul and Sharma, 2004) and its antibacterial properties (El-Sharif and Hussain, 2011). The % wound closure of all of the treatments until recovery was similar, which suggests that wound healing was progressed by mechanism of the body that is independent from the effects of the treatment. This result is related to a previous study, the lysozyme loaded chitosan-based nanofiber mats, which exhibited better wound healing activity than gauze at 1–4 days after treatment (Charernsriwilaiwat et al., 2012a).

The histological examination images of the skin wounds treated with different dressings at 11 days after initial operation by hematoxylin and eosin are shown in Fig. 8. In normal skin, the bundles of collagenous fibers are loose and wavy with few fibroblasts. During wound repair, fibroblasts play an important role through karyokinesis and proliferation, and synthesizing and secreting substantive collagenous fibers over the first 4 to 6 days, creating aceresoma tissue with new capillary vessels. This process fills the wound with granulation tissue and provides the covering conditions for new epidermis (Liu et al., 2010). The wound treated with gauze, commercial antibacterial gauze dressing and the 0 wt% α-mangostin nanofiber mats were completely reepithelialized. However, the wound treated 3 wt% α-mangostin nanofiber mats were completely reepithelialized and almost healed, and the granulation tissues were nearly replaced by hair follicles. This result indicated that the CS-EDTA/PVA nanofiber mats loaded with GM extracts accelerated the healing rate.

3.8. Stability studies

The 3 wt% α-mangostin nanofiber mat was stored under normal conditions (25°C 40%RH) and with a sample stored under stress conditions (45°C 75%RH) for 6 months. Fig. 9 shows the
appearance and the SEM images of the mats at initial, 3 and 6 months after storage in different conditions. The appearance of the mats maintained under the stress condition exhibited more color change (dark yellow) than those stored under normal conditions (pale yellow). However, the morphology observed using SEM images of the mats maintained under both conditions was similar. The fibers were smooth and did not contain crystals of the GM extracts in their structure after storage in either condition for 6 months. The physical and chemical stability of the mats are shown in Fig. 10. The average diameter and remaining α-mangostin (%) in the mats during storage under normal and stress conditions were not statistically different. The diameters of the mats after storing for 3 and 6 months were in the range of 250–260 nm. The amount of α-mangostin remaining in the fiber mats was decreased from 90% in the first month to 65% after 6 months. The α-mangostin was stable under light, heat and basic hydrolysis (Yodhnu et al., 2009). Pothitirat et al. (2009b) reported that the G. mangostana fruit rind extract maintained the content of α-mangostin, antiradical and anti-acne when kept at different temperature (4–8, 25–28 and 45 °C) for 120 days (Pothitirat et al., 2009b). This result corresponds to current result, where the content of α-mangostin in the nanofiber mats remained at approximately 90% for 3 months. However, the antioxidant activity and the remaining tensile strength (%) of the mats stored under stress conditions exhibited worse properties than the mats stored under normal conditions. The results obtained from this stability study led us to assume that the better stability of the GM loaded fiber mats is the consequence of the lower temperature storage.

Fig. 8. Histological images of the wound skins at 11 days after treated with different wound dressing: (a) control (untreated skins), (b) gauze (negative control), (c) commercial antibacterial gauze dressing (Sofra-tulle®) (positive control), (d) 0 wt% α-mangostin and (e) 3 wt% α-mangostin GM extracts loaded CS-EDTA/PVA nanofiber mats.

Fig. 9. The SEM image and the appearance of the 3 wt% α-mangostin GM extracts loaded CS-EDTA/PVA nanofiber mats when stored under normal conditions (a, b and c) (25 °C 40%RH) compared with stress conditions (d, e and f) (45 °C 75%RH) for 0, 3 and 6 months, respectively.
4. Conclusion

In the present study, GM extracts were incorporated into CS-EDTA/PVA nanofiber mats using the electrospinning process. The fiber mats provided suitable tensile strength and swelling properties. These mats are non-toxic and α-mangostin is rapidly released, which retains the antioxidant and antibacterial activity, and accelerates the wound healing process. These biodegradable, biocompatible and antibacterial electrospun nanofiber mats have promising potential for use as effective wound dressings.

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