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Mucoadhesive electrospun chitosan-based nanofibre mats for dental caries prevention

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ABSTRACT

The mucoadhesive electrospun nanofibre mats were developed using chitosan (CS) and thiolated chitosan (CS-SH) as mucoadhesive polymers. *Garcinia mangostana* (GM) extract was incorporated into nanofibre mats. The antibacterial activity in the single and combined agents was evaluated against dental caries pathogens. The morphology of mats was observed using SEM. The mats were evaluated for GM extract amount, mucoadhesion, in vitro release, antibacterial activity and cytotoxicity. The mucoadhesion and antibacterial activity were determined in healthy human volunteers. The prepared mats were in nanoscale with good physical and mucoadhesive properties. The CS-SH caused the higher mucoadhesion. All mats rapidly released active substances, which had the synergistic antibacterial activity. In addition, the reduction of bacteria and good mucoadhesion in the oral cavity occurred without cytotoxicity. The results suggest that mats have the potential to be mucoadhesive dosage forms to maintain oral hygiene by reducing the bacterial growth that causes the dental caries.

1. Introduction

The buccal mucosa has excellent accessibility, an expanse of smooth muscle and relatively immobile mucosa, hence suitable for administration of retentive dosage forms (Sudhakar, Kuotsu, & Bandyopadhyay, 2006). However, the effect of salivary scavenging and accidental swallowing can potentially lead to loss drug (Li & Robinson, 2005). Therefore, the polymers as mucoadhesive materials are required to retain a dosage form. Chitosan (CS) is a cationic material that has been proposed as mucoadhesive polymer that will bind to mucins via electrostatic interactions (Morales & McConville, 2011; Sudhakar et al., 2006). Non-ionic polymers like polyvinyl alcohol (PVA) also show the adhesive force due to the forming of hydrogen bonding. However, these polymers adhere to the mucus non-specifically and suffer short retention times. These lead to synthesise the newer polymers that exhibit free thiol groups on the polymeric backbone which can form disulfide bonds with cysteine residues of mucins, contributing the interactions for mucoadhesion (Langoth, Kalbe, & Bernkop-Schnürch, 2003; Marschütz & Bernkop-Schnürch, 2002).

Currently, the electrospun nanofibre mats has been attractive for mucoadhesive formulations. The electrospinning process appears to be the method that can be developed as one continuous step of mats. One of the application of mucoadhesive formulations, they can be used the prevention and treatment of oral diseases. *Streptococcus mutans* (*S. mutans*) is a major causative agent for dental caries. It is also with *Streptococcus sanguinis* (*S. sanguinis*) and *Lactobacillus* spp., which is a member of the pioneer species in dental plaque formation (Frandsen, Pedrazzoli, & Kilian, 1991; Hotta, Nakajima, Yamamoto, & Aono, 1998). Hence, eradication of these bacteria is importance for the prevention of dental caries. CS does not have only mucoadhesive property but also antibacterial activity. The pericarp of mangosteen (*Garcinia mangostana*, GM) is one of the most used traditional medicines. GM pericarp contains a variety of xanthones, benzophenones, flavonoids and tannins which have pharmaceutical functions (Pedraza-Chaverri, Cárdenas-Rodríguez, Orozco-Ibarra, & Pérez-Rojas, 2008). Our previous study reported the antioxidative activity and antibacterial activity of GM to promote wound healing effect (Charernsriwilaiwat, Rojanarata, Ngwhirunpat, Sukma, & Opanasopit, 2013). However, there is a lack of scientific reports that indicate the antibacterial activities against oral pathogens, especially for dental caries pathogens. Moreover, the previous study did not report the synergistic activity of CS with

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GM and the mucoadhesive property of CS and CS derivatives. Thus, this study focused on the preparation of mucoadhesive electrospun chitosan-based nanofibre mats loaded with GM extract to improve antibacterial activity. The efficacy of antibacterial combination was examined. Thiolated chitosan (CS-SH) was synthesised and used as a mucoadhesive polymer. The CS or CS-SH with ethylenediaminetetraacetic acid (EDTA) and PVA was prepared as polymer base. The nanofibre mats were evaluated for properties that included GM extract amount, swelling, mucoadhesion, in vitro release, antibacterial activity and cytotoxicity. In vivo study was also performed in healthy human volunteers.

2. Materials and methods

2.1. Materials

The pericarp of GM was obtained from a farm in Chantaburi Province, Thailand. CS (degree of deacetylation 0.85, MW 110 kDa), EDTA, cysteine hydrochloride, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) 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. All other reagents and solvents were of analytical grade.

2.2. GM extraction

The GM pericarp was macerated with 95% ethanol until the extraction was exhausted. The extract was filtered through a Whatman no. 1 filter paper under suction. The filtrate was evaporated solvent with a vacuum rotary evaporator.

An active constituent α-mangostin was determined using HPLC (Agillent 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, Chomnawang, Supabphol, and Gritsanapan (2009). The content of total phenolic compounds was determined using Folin–Ciocalteu procedure (Singleton, Orthofer, & Lamuela-Raventós, 1999), which was calculated and expressed as milligrams of gallic acid equivalents (GAE)/g of GM extract. The content of total tannins was assayed by protein precipitation method (Silber & Fellman, 2006), which was calculated and expressed as milligrams of tannic acid equivalents (TAE)/g of GM extract.

2.3. Antibacterial activity of substances

The antibacterial activity of GM extract, CS, EDTA and CS-EDTA was tested against *S. mutans* A32–2 and *S. sanguinis* ATCC 10556 using a broth dilution technique (Jorgensen & Turnidge, 2007). The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined. The GM extract, CS and EDTA were dissolved in dimethyl sulfoxide (DMSO), 0.5% acetic acid and distilled water, respectively. The CS-EDTA was prepared by dissolving CS and EDTA in distilled water at a weight ratio of 2:1. The serial two fold dilutions of samples were prepared with brain heart infusion (BHI) broth. The culture of bacteria was added and adjusted to $1–2 \times 10^6$ colony-forming units (CFU)/ml. After 24 h incubation, the bacterial growth was observed for turbidity. The MIC was defined as the lowest concentration that presented in the clear well. An aliquot from dilution tubes without turbidity was inoculated on BHI agar plates. The plates were then incubated for 24 h. The MBC was defined as the lowest concentration that reduced the number of viable bacteria by 99.9% (Hindler, 2000). BHI broth with bacterial suspension and solvent served as the negative controls and 0.2% w/v chlorhexidine was used as the positive control.

A checkerboard assay was used to evaluate the antibacterial efficacy of the combination of CS with EDTA and CS-EDTA with GM extract. The method used was previously described by White, Burgess, Manduru, and Bosso (1996). The fractional inhibitory concentration (FIC) was calculated for each combination. The FIC index is FIC of A + FIC of B where FIC of A is MIC of A in combination/MIC of A alone, and FIC of B is MIC of B in combination/MIC of B alone. The FIC was interpreted as synergistic when it is ≤0.5 and antagonistic when it is >4; a FIC result of >0.5 but ≤4 is indifferent.

2.4. Synthesis of thiolated chitosan (CS-SH)

CS was dissolved in 1% v/v HCl. Additionally, cysteine was dissolved in deionised water. The carboxylic acid groups of cysteine were activated with 150 mM EDAC for 20 min. The pH was adjusted within the range of 4–5 and maintained during the experiment. After stirring for 6 h, the CS-SH was isolated by dialysing for 3 days. The sample was frozen and dried at −49 °C and 0.07–0.09 bar (Freezone 2.5, Labconco, UK). The amount of thiol group was determined with Ellman's reagent. First, 50 μl of Ellman's reagent (4 mg of DTNB dissolved in 1 ml of 0.1 M phosphate buffer pH 8 and 1 mM EDTA), and 2.5 ml of 0.1 M phosphate buffer pH 8 and 1 mM EDTA was added to the test tube. The 250 μl of standard cysteine and sample were added and mixed together. All tubes were incubated for 15 min before measurement absorbance at 412 nm. Cysteine standards were used to calculate the amount of thiol groups immobilised on the CS-SH.

2.5. Preparation of GM extract loaded nanofibre mats

The 2% w/v CS or CS-SH solution was prepared by dissolving CS or CS-SH with EDTA in distilled water at the weight ratio of 2:1. The 10% w/v PVA solution was prepared by dissolving PVA in distilled water at 80 °C and then allowing the solution to stir for 4 h. The CS or CS-SH solution was mixed with PVA solution at weight ratios of 30:70. GM extract (containing 1, 3 and 5% w/w α-mangostin to polymer) was added to CS/PVA or CS-SH/PVA solution by stirring for 24 h. The solution's viscosity, conductivity and surface tension were measured before preparing the mats via the electrospinning process. The solution was contained in a glass syringe connected with a 20-gauge, stainless steel needle (diameter = 0.9 mm) at the nozzle. The needle was connected to the emitting electrode of positive polarity of a Gamma High Voltage Research device (Ormond Beach, Florida, USA). The electric potential was fixed at 15 kV. The nanofibre mats were collected as-spun on an aluminium foil that was covered on a rotating collector. The collection distance was fixed at 20 cm. The solution feeding rate was fixed at 0.45 ml/h using the syringe pump (Model: NE-300, New Era Pump Systems Inc.).

2.6. Characterisations of nanofibre mats

The morphology and diameter of nanofibre mats were examined using a SEM (Camscan Mx2000, England). The average diameter of mats was determined using image analysis software (JMicroVision V.1.2.7, Switzerland); fifty measurements were collected from the SEM image.

The powder X-ray patterns of nanofibre mats were recorded using an X-ray powder diffractometer (model miniflex II, Rigaku Co., Japan). The sample was irradiated with monochromatised CuKa radiation after passing through Nickel filters and then was analysed between 5° and 45° (2θ). The voltage and current applied were 30 kV and 15 mA, respectively.

2.7. Evaluations of nanofibre mats

2.7.1. The GM extract content

The amount of GM extract in a mat was determined by submerging the mat in methanol for 24 h. Then, the solution was analysed using HPLC to determine the amount of α-mangostin. The loading efficacy (%) of GM extract was calculated, which was the amount of GM extract that was embedded in the mat/the theoretical amount of GM extract.

2.7.2. Mucoadhesive study

The mucoadhesive study was performed according to a previous study (Thirawong, Nunthanid, Puttipipatkhachorn, & Sriamornsak, 2007). The mucoadhesion of mats onto porcine buccal mucosa was carried out by a texture analyser with a 50 N load cell equipped with a mucoadhesive holder. The nanofibre mat was cut and attached to the cylindrical probe. The tissue was equilibrated at 37 ± 0.5 °C and placed onto the holder stage of mucoadhesive holder. Then, artificial saliva was added onto the buccal mucosa. The probe with the mat was put in contact with the buccal mucosa with 3 g of force for 1 min, and the mat was then withdrawn. The maximum force required to separate the probe with the mats from the tissue was recorded using Texture Exponent 32 software.

2.7.3. In vitro release of GM extract

The GM extract release was investigated in the mixture solvent of artificial saliva and methanol (50:50). The mats were placed into the release medium. The medium was maintained at 37 ± 0.5 °C with a speed of 150 rpm throughout the test. At a given time point, a sample solution was withdrawn and an equal amount of fresh medium was refilled. The amount of GM extract in the sample solutions was analysed by HPLC.

2.7.4. Antibacterial activity of nanofibre mats

The mats were sterilised by UV radiation for 1 h before evaluation of antibacterial activity against *S. mutans* and *S. sanguinis* and then were put onto the bacterial suspensions. The MIC and MBC were determined as described in Section 2.3. The rate of bactericidal activity was examined by a time kill assay. Bacterial suspensions were treated with mats at a fixed concentration of MBC of blank mats. At the indicated time of incubation, sample was collected, diluted and spread on BHI agar plates. The numbers of viable bacteria were counted after incubation for 48 h. The cultivation of bacteria was used as a control for bacterial growth at each time point. The graph of time against the numbers of viable colonies was plotted.

2.8. Cytotoxicity

The cytotoxicity of mats was evaluated using the human keratinocyte cell line (HaCaT) and human gingival fibroblasts (HGF). HGF were obtained from explants of gingival tissue attached to non-carious, freshly extracted third molars. Ethical approval for the study had been obtained from Naresuan University Institutional Review Board (COE No. 55 02 04 0024). The mats were sterilised by UV radiation for 1 h before testing. Each cell was seeded onto the 48-well plates. When the cultures reached confluence, the serum free medium (SFM) was replaced, and the mats were put onto the wells. The cells were incubated for 15, 30, 60, 120 and 240 min. The tests also determined the long term cytotoxicity by rinsing the cells with phosphate buffer pH 7.4 at the end of testing. Then, the SFM was replaced and re-incubated for 24, 48 and 72 h. After treatment, the cytotoxicity was examined by MTT assay. The cell viability (%) was calculated based on the absorbance at 570 nm using a Genesis10 UV-vis spectrophotometer (Thermo Spectronic, NY, USA). The viability of the non-treated control cells was arbitrarily defined as 100%.

2.9. In vivo study

An in vivo study was performed with healthy human volunteers to evaluate the antibacterial activity, the mucoadhesive property, the taste and the mouth feeling of mats. A blind test was chosen for this study. This study was approved by an Investigational Review Board (Human Studies Ethics Committee, Faculty of Pharmacy, Silpakorn University, approval no.9-2556). Each volunteer took one mat and then adhered to the buccal mucosa. The time period of adhesion was recorded as the in vivo adhesion time. Non-stimulated saliva samples were collected using the spitting method at baseline, 15, 30 and 60 min. The bitterness and mouth feel were observed during the test. After the test, the volunteers rinsed their oral cavities with water without swallowing the disintegrated material. The saliva was reserved for microbiological measurements by dilution and spread on the mitis salivarius agar and lactobacillus MRS agar. After incubation of the samples, the total number of CFU/ml was determined. The number of bacteria at baseline was arbitrarily defined as 100%.

2.10. Statistical analysis

All experimental measurements were collected in triplicate. Data were expressed as the mean ± SD. The statistical significance of the difference in numbers of viable bacteria of time kill assay and % cell viability of cytotoxicity test were examined using one-way analyses of variance (ANOVA) and Student's *t*-test, respectively. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Standardisations of GM extracts

The maceration of GM with ethanol was selected to extract the active compounds. This was due to the ability of ethanol to penetrate the sample during extraction. This ethanolic extraction had good efficiency in degrading the plant cell and promoted a greater amount of endocellular material (Pothitirat et al., 2009). The extraction yield was 18.97 ± 2.69%. The amount of α–mangostin, phenolic compounds and tannins was 131.52 ± 0.65 mg/g extract, 481.19 ± 5.51 mg GAE/g extract and 158.77 ± 1.08 mg TAE/g extract, respectively. These findings confirmed that the ethanol extraction was composed of the active substituents before loading into the mats.

3.2. Antibacterial activity of substances

The low MIC and MBC of GM extract, CS, EDTA and CS-EDTA solutions against *S. mutans* and *S. sanguinis* (Table S1) indicated that these compounds had strong antibacterial activity. The GM extract had the best activity. The mechanism of α-mangostin was targeted to the cell membrane; it induced a rapid dissipation of membrane potential and subsequently caused considerable leakage of intracellular components (Koh et al., 2013). The phenolic compounds caused the rapid disruption of cell membrane and non-ionic surfactant type of action which was disrupted at the lipid-protein interface (Greenberg, Dodds, & Tian, 2008). Tannins had astringent and protein precipitation properties that could induce complexation with proteins, enzymes and metal ions of bacteria (Akiyama, Fujii, Yamasaki, Oono, & Watsuka, 2001). This complexation inhibited the growth of bacteria by the impediment of vital functions, including the ribonucleotide precursor of DNA and the cell division process.

Table 1
Solution parameters of GM extract loaded CS/PVA and CS-SH/PVA solution and loading efficiency of GM extract loaded CS/PVA and CS-SH/PVA nanofibre mats.

GM extract (%w/w α-mangostin)	Solution parameters						GM extract loaded nanofibre mats	
	Viscosity (mPas)		Conductivity (μS/cm)		Surface tension (mN/m)		Loading efficiency (%)	
	CS/PVA	CS-SH/PVA	CS/PVA	CS-SH/PVA	CS/PVA	CS-SH/PVA	CS/PVA	CS-SH/PVA
0	164.5 ± 1.6	230.4 ± 0.7	867.3 ± 52.3	2363.2 ± 55.1	55.2 ± 0.9	54.5 ± 1.2	–	–
1	181.3 ± 1.2	232.1 ± 3.2	779.0 ± 20.1	1933.0 ± 16.5	54.2 ± 0.6	49.4 ± 0.1	70.3 ± 1.4	69.7 ± 0.8
3	191.9 ± 4.6	253.2 ± 0.7	798.7 ± 38.2	1855.3 ± 24.4	47.1 ± 1.9	46.6 ± 0.1	74.9 ± 0.1	61.9 ± 0.1
5	190.3 ± 0.5	267.1 ± 0.7	811.3 ± 7.6	1773.0 ± 9.5	45.0 ± 0.8	44.5 ± 0.1	79.7 ± 0.1	73.1 ± 0.5

The second antibacterial activity was CS-EDTA. EDTA promoted the CS solubility by forming a complex with CS. In addition, EDTA also enhanced the antibacterial activity. The CS-EDTA exhibited the lower MIC and MBC compared with CS or EDTA alone. The mechanism of CS involved the cationic charge of amino group, which interfered with the anionic component of the bacterial cells (El-Sharif & Hussain, 2011). EDTA was inhibited the growth of bacteria by inhibiting enzymes that require cations as cofactors (Banin, Brady, & Greenberg, 2006). The effective combination of CS and EDTA was confirmed by a checkerboard assay. The lower of 0.5 of FIC index (Table S2) demonstrated the synergistic antibacterial activity. Furthermore, this assay indicated that CS-EDTA and GM extract were synergistic due to the FIC index (Table S2).

3.3. Electrospinning process for nanofibre mats

The CS/PVA and CS-SH/PVA (469.8 ± 2.8 μmol of free thiol groups per gram of CS-SH) solutions containing an equivalent amount of GM extract were prepared and measured the viscosity, conductivity and surface tension before preparing the mats, as shown in Table 1. The CS-SH/PVA solution had a higher viscosity and conductivity compared with the CS/PVA solution, while the surface tension was not different. This was due to the greater viscosity of CS-SH (Mueller, Verroken, Iqbal, & Bernkop-Schnürch, 2013). The conjugation with cysteine might increase the polarity of CS; thus, CS-SH could easily conduct an electric current. The viscosity and conductivity of the solution had a significant effect on the mats's morphology, as shown in Fig. 1. When the viscosity was increased, the nanofibre diameter also increased (Zong et al., 2002). However, the increased conductivity caused the decrease in the diameter. The CS-SH/PVA solution was three times higher in conductivity compared with CS/PVA, which indicated the effect of conductivity was dominant than the viscosity. The CS-SH/PVA mat was smaller compared with the CS/PVA mats (Fig. 1a and e). When the GM extract was added, the diameter increased due to the increased viscosity and reduced conductivity of the solution. The results of both composite solutions were corresponding. However, the CS-SH/PVA nanofibres remained smaller in the diameter compared with the CS/PVA nanofibres.

3.4. Characterisations and evaluations

The X-ray pattern of the α-mangosin powder and the nanofibre mats are shown in Fig. 2a and b. The α-mangostin was crystalline and exhibited a number of sharp peaks, which indicated the crystalline nature. The blank mats showed the hollow pattern, which indicated the amorphous state of mats. When the GM extract was incorporated into either mat, the mats showed the hollow pattern and the absence of α-mangostin peaks, which indicated that α-mangostin was converted from a crystalline into an amorphous state.

The amount of GM extract in the mats was determined as the loading efficacy, which is shown in Table 1. The GM extract could be loaded in the mats, which were approximately 70%. The GM extracts are slightly soluble in water, but they are soluble in a

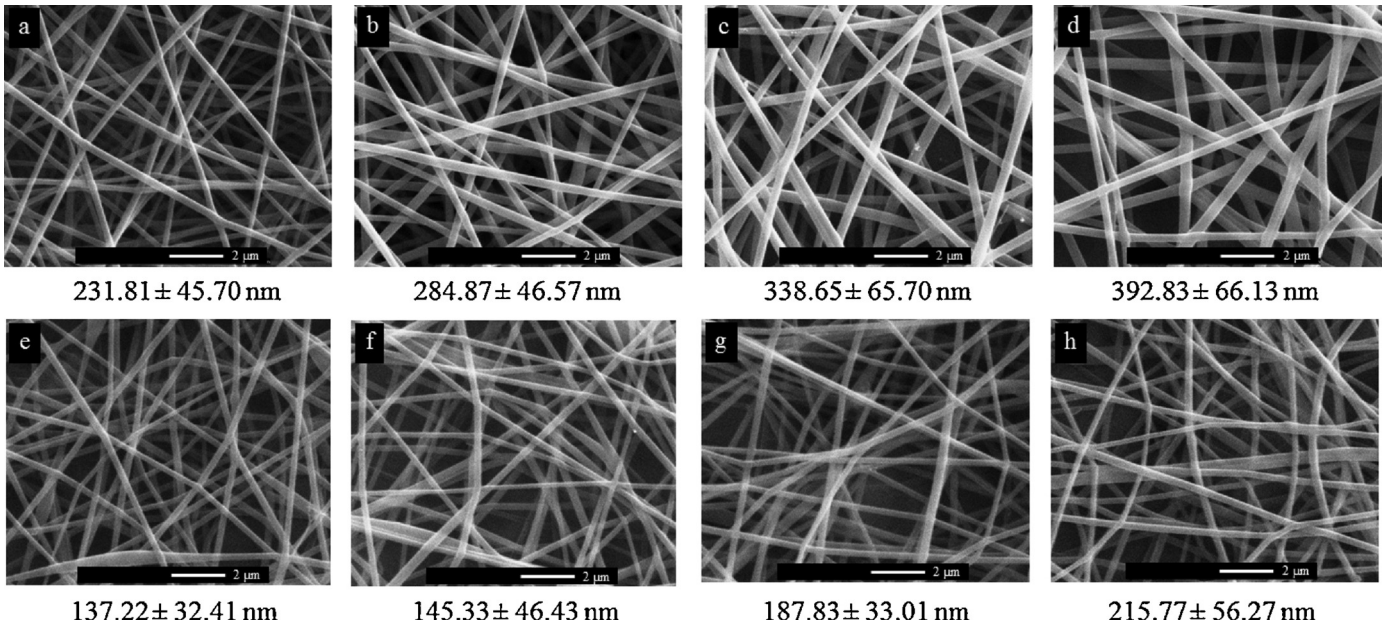

Fig. 1. The SEM image and diameter of the GM extract loaded (a–d) CS/PVA and (e–h) CS-SH/PVA nanofibre mats with different amounts of GM extract: (a and e) 0, (b and f) 1, (c and g) 3 and (d and h) 5% w/w α-mangostin.

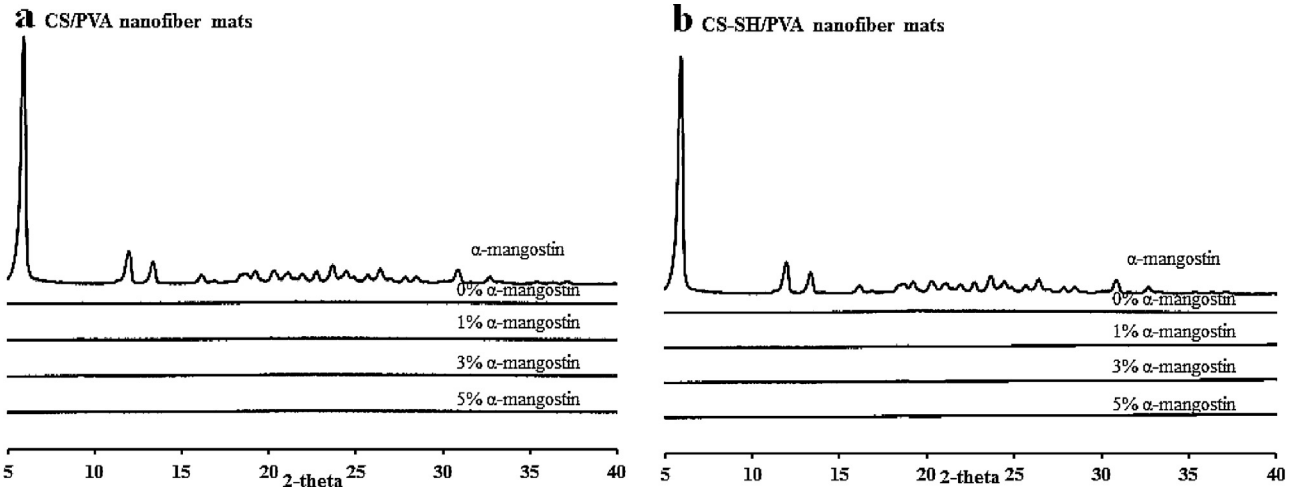

Fig. 2. X-ray patterns of α-mangostin and the GM extract loaded (a) CS/PVA and (b) CS-SH/PVA nanofibre mats with the different amounts of GM extract (0%, 1%, 3% and 5% w/w α-mangostin).

variety of non-polar solvents (Yichu & Zhang, 2010), causing the limited incorporation of GM extract into the mats.

The force required for the detachment of the CS-SH/PVA mats from porcine buccal mucosa was two times (~22 g) higher compared with the CS/PVA mats (~11 g). These findings revealed that CS-SH significantly improved the mucoadhesive property. The added GM extract did not affect the mucoadhesive property in both type mats. The mucoadhesive force was constant when the GM extract was increased.

Fig. 3 shows the α-mangotin release profiles from the mats. There were no differences in the release profiles between the CS/PVA and CS-SH/PVA mat. All mats rapidly released approximately 80% of α-mangostin within 60 min. The complete release was occurred within 240 min. This result indicated that the burst release was occurred due to the increase in surface area and the porosity of nanofibre mats. The α-mangostin in the GM extract could release via diffusion, as well as the degradation of polymer carriers (Sill & Recum, 2008).

3.5. Antibacterial activity of nanofibre mats

Table 2 exhibits the MIC and MBC of mats against *S. mutans* and *S. sanguinis*. The mats containing GM extract displayed a concentration dependent antibacterial activity when GM extract was increased, indicating that the potency was dependent on the amount of GM extract. The blank mats also inhibited and destroyed bacteria. This effect was due to the antibacterial activity of CS, CS-SH and EDTA, as previously described. The CS-SH/PVA mats exhibited a slightly weaker activity compared with the CS/PVA mats. The conjugation of CS reduced the free cationic charge of amino groups that interfered with the anionic component of the bacterial cells (Han, Wei, Jia, Xu, & Li, 2012). However, the amino group was not completely substituted, and there were some amino groups that could interact with the bacterial cells. When the mats were submerged into the bacterial suspension, the GM extract, CS, CS-SH and EDTA was released. Finally, these compounds actively presented the synergic activity against bacterial cells.

The time kill assay of a fixed concentration at the MBC of blank mat was studied to elucidate the effect of GM extract on the rate of bactericidal activity. The time kill curves of mats are presented in Fig. 4. The results of CS/PVA and CS-SH/PVA mats for each strain were similar, and the viable cells led to a rapid decrease in the bacterial number with an increasing exposure time. This finding was in contrast with the cultivation of bacteria, in which the bacteria swiftly divided themselves and the number of cells rapidly increased. The killing was time-dependent and related to the release of GM extract. When the incubation time increased, the amount of GM extract, CS, CS-SH and EDTA was increased, and

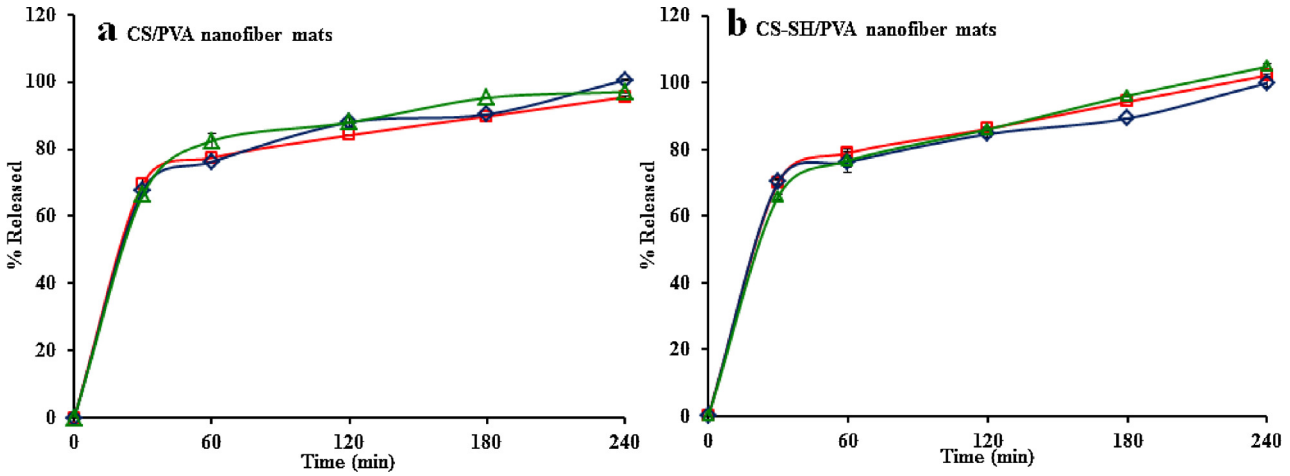

Fig. 3. The α-mangostin release profiles from (a) CS/PVA and (b) CS-SH/PVA nanofibre mats containing the various amounts of GM extract: (□) 1, (◇) 3 and (△) 5% w/w α-mangostin.

Table 2
Antibacterial activity of CS/PVA and CS-SH/PVA nanofibre mats containing the GM extract against *S. mutans* and *S. sanguinis*.

GM extract (%w/w α-mangostin)	MIC (mg/ml)				MBC (mg/ml)			
	S. mutans		*S. sanguinis*		*S. mutans*		*S. sanguinis*	
	CS/PVA	CS-SH/PVA	CS/PVA	CS-SH/PVA	CS/PVA	CS-SH/PVA	CS/PVA	CS-SH/PVA
0	2	3	1	1	3	4	2	3
1	0.5	1	0.5	0.5	2	2	1	2
3	0.2	0.5	0.2	0.2	1	1	0.5	1
5	0.1	0.2	0.1	0.1	0.5	0.5	0.2	0.5

the interaction between the active agents and the bacterial cells was also increased. These incidents motivated the numbers of cell deaths and reduced the number of vital bacteria. The increased GM extract in the mats led to a more rapid decrease in the bacterial number. For *S. mutans* (Fig. 4a and b), the fast killing rate was observed at 30, 60 and 120 min. The viable cell numbers of the higher GM extract mats were significantly lower compared with the mats that contained less GM extract, the blank mats and the control bacteria at 30 min. At 60 and 120 min, the viable cell number was also decreased, and was significantly smaller compared with the blank mats and the control, but there were no significant difference between the various amounts of GM extract. At the other time points (180 and 240 min), the number of *S. mutans* was significantly reduced from the control; however, it was not significantly different between each mat, and the cells were killed by all mats within 240 min. A similar trend was observed for *S. sanguinis* (Fig. 4c and d), and the loaded GM extract was characterised by a rapid killing rate within 30 min. All *S. sanguinis* were eliminated within 60 min.

3.6. Cytotoxicity

The results of the acute cytotoxicity are shown in Fig. 5. There was no difference in cytotoxicity between CS/PVA and CS-SH/PVA mats. For the HaCaT (Fig. 5a and b), no significant difference in cell viability was observed between blank mats and control within 240 min. However, there was a significant decrease in the cell viability at the longer time point when the mats contained GM extract compared with the control. The HaCaT viability was significantly decreased at 240, 120 and 120 min when the cells were treated with 1%, 3% and 5% α-mangostin of GM extract loaded mats, respectively. These results indicated that the HaCaT cells existed for 240, 120, 60 and 60 min in the 0%, 1%, 3% and 5% α-mangostin of GM extract loaded mats, respectively. These results might be because of the amount of GM extract. When the incubation time increased, the higher release in the GM extract interacted with the cells and then caused cell death by an apoptosis mechanism (Nakagawa, Iinuma, Naoe, Nozawa, & Akao, 2007). The results of cytotoxicity on the HGF (Fig. 5c and d) were similar to the HaCaT results, and

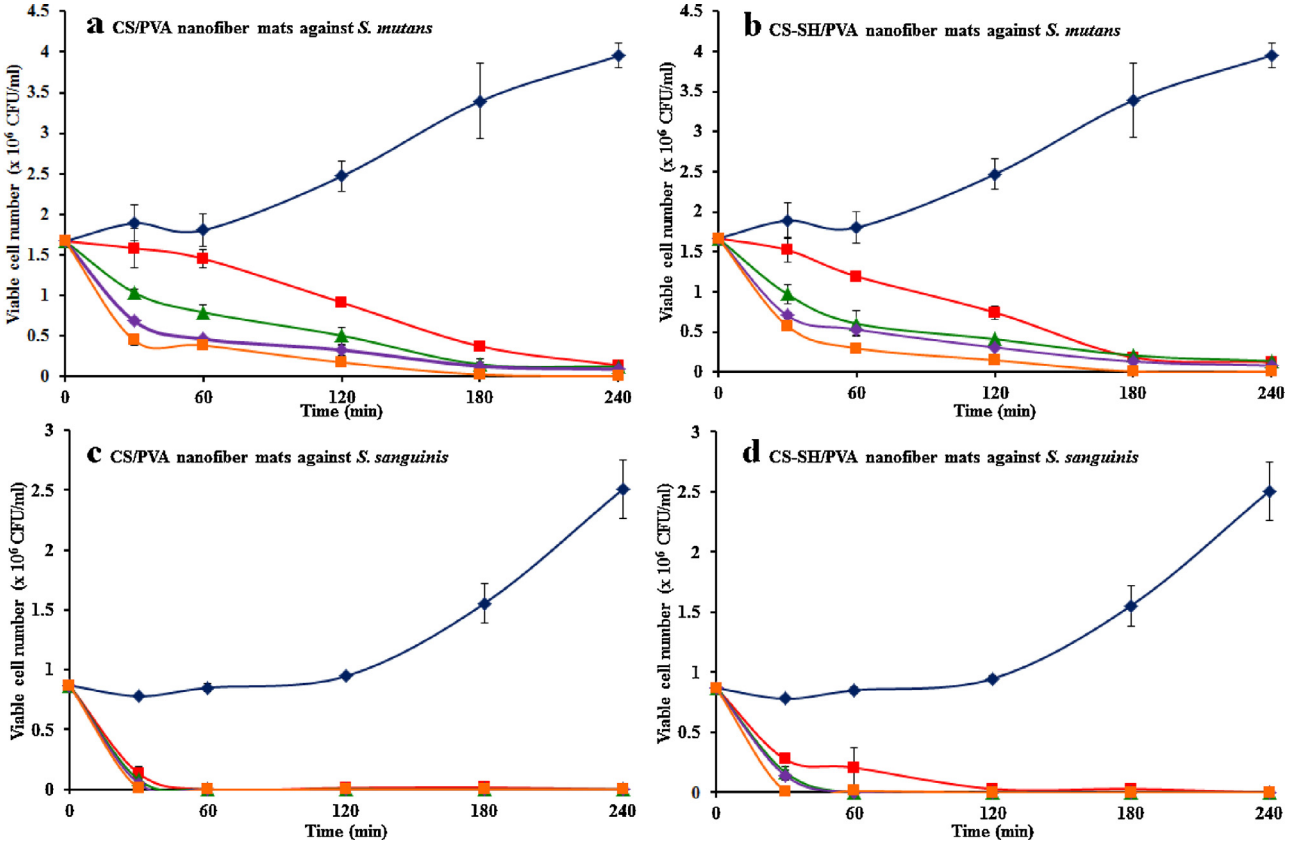

Fig. 4. Time kill curve of *S. mutans* and *S. sanguinis*: (◆) untreated (control) and treated with the GM extract loaded CS/PVA and CS-SH/PVA nanofibre mats with the different amounts of GM extract: (■) 0, (▲) 1, (◆) 3 and (■) 5% w/w α-mangostin.

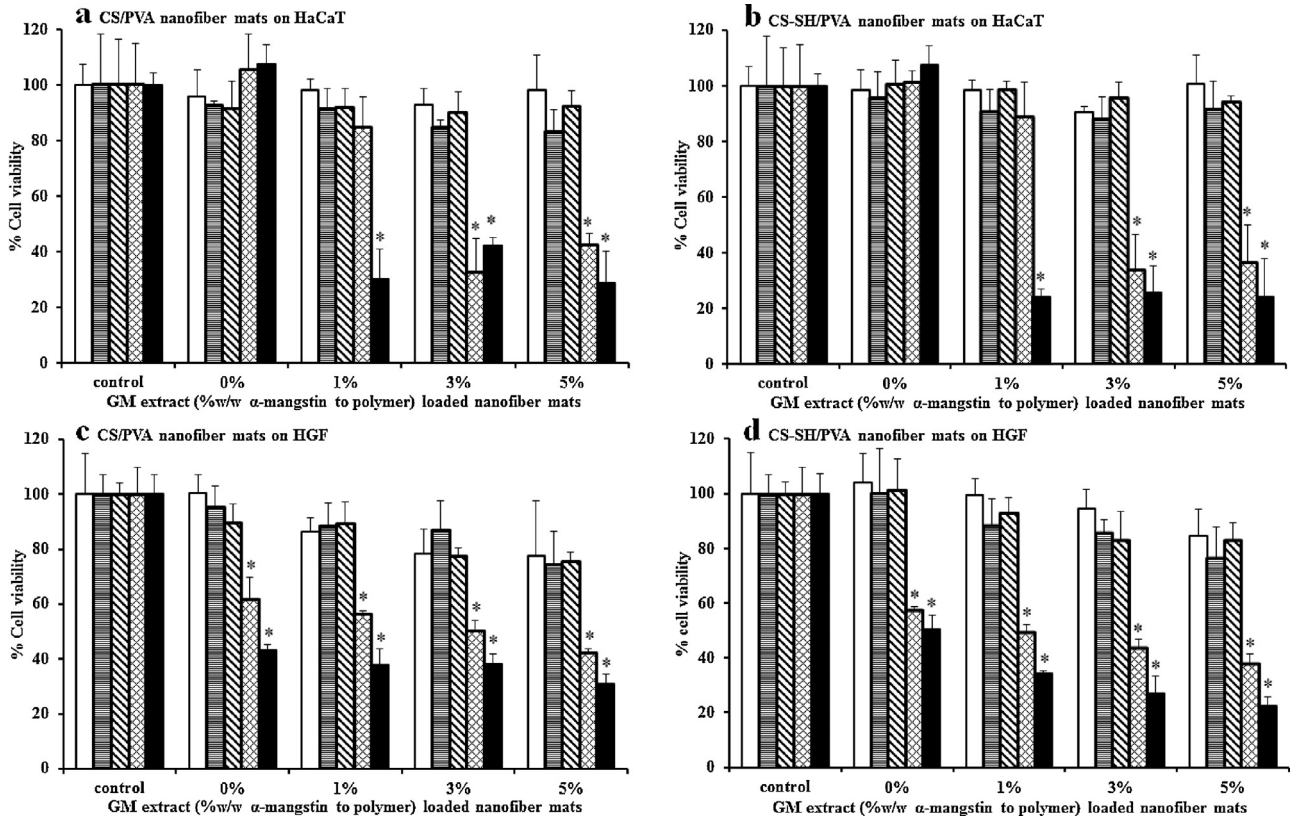

Fig. 5. The percentage of cell viability in HaCaT and HGF for acute cytotoxicity at different amounts of GM extract loaded (a and c) CS/PVA and (b and d) CS-SH/PVA nanofibre mats: incubation for (□) 15, (▤) 30, (▨) 60, (■) 120 and (■) 240 min. * statistically significant ($p < 0.05$).

the reduction of cell viability was observed in the longer time. There was a significant decrease in the cell viability when HGF were incubated with all mats at 120 and 240 min compared with control. However, HGF viability was not significantly different from the control at the times of 15, 30 and 60 min. The cell viability was more than 80% at these times. These experiments using both cells indicated that cytotoxicity of both cells was different. The HGF were more sensitive compared with HaCaT because they are primary cells. Thus, the cytotoxicity depended on the toxicity of HGF. All mats could be safely used in the oral cavity for 60 min. The long term cytotoxicity was also determined by removal of medium and rinsing the treated cells with phosphate buffer solution after 60 min of an acute cytotoxicity test. The cell viability of both cells was not significantly different from the control at 24, 48 and 72 h of the re-incubation for all mats (Fig. S1). The results indicated that the use of the mats for 60 min was safe and less cytotoxic for 72 h.

3.7. *In vivo study*

The 1% α-mangostin of GM extract loaded mats was selected as the optimal mat to study in vivo due to its safety and activity within 60 min. The CS-SH/PVA mat (6.44 ± 2.49 min) provided a longer adhesion time compared with the CS/PVA mat (1.94 ± 0.56 min). This result corresponded to an in vitro study. The bitterness was not observed in both mats. Additionally, the smoothness was measured

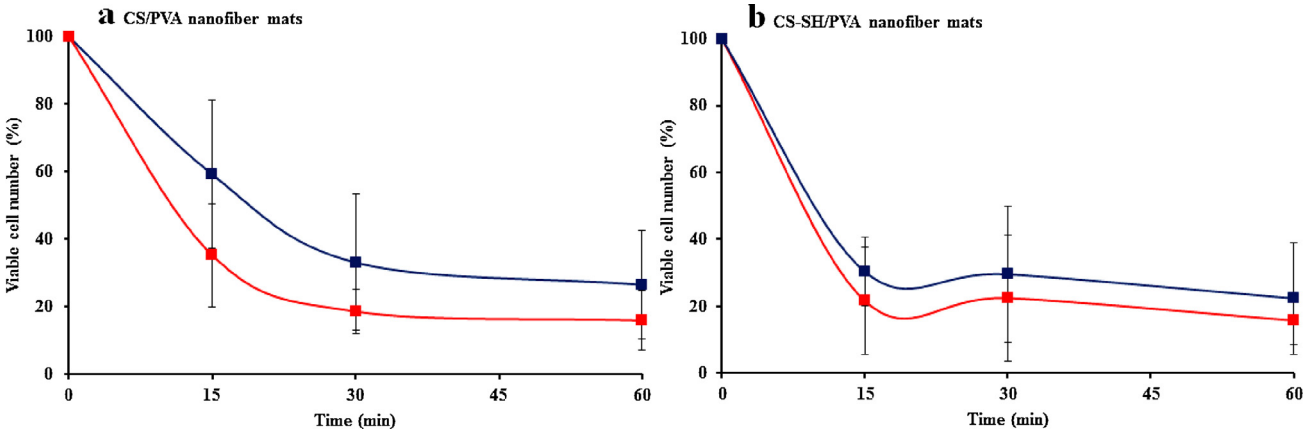

Fig. 6. In vivo antibacterial activity of 1% w/w α-mangostin of GM extract loaded (a) CS/PVA and (b) CS-SH/PVA nanofibre mats against oral bacteria: (■) *Streptococcus* spp. and (■) *Lactobacillus* spp.

during the adhesion of mats. These good properties illustrated that the mats may be gladly used by patients.

The in vivo antibacterial activity of mats determined that mats had an antibacterial effect in the oral cavity (Fig. 6). Both mats did not show any difference in antibacterial activity. The number of bacteria was significantly reduced during the three sampling times. After a mat was adhered to buccal mucosa, the CS, CS-SH and PVA began to swell, and the active substituents were released to react with oral bacteria. Then, a mat was detached from the buccal mucosa, and the antibacterial activity continued for 60 min. The 70% and 80% reduction in *Streptococcus* spp. and *Lactobacillus* spp. were found after the application of mats. This result indicated that the mats not only reduced the in vitro bacteria but also the in vivo oral species.

4. Conclusion

GM extract loaded CS/PVA and CS-SH/PVA mucoadhesive nanofibre mats were successfully prepared using an electrospinning process. The mats were smooth in the nanometre range with good properties of mucoadhesion. These mats rapidly released the active substances, which had excellent synergistic antibacterial activity on dental caries pathogens. The non-toxicity of mats could be safely used with good taste and feeling in the oral cavity, which reduced the number of oral pathogens. Thus, these mats have the potential to be good choices to maintain oral hygiene by reducing the bacterial growth in saliva, which causes the development of dental caries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.10.026.

References

Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., & Watsuka, K. I. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, *48*, 487–491.

Banin, E., Brady, K. M., & Greenberg, E. P. (2006). Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Applied and Environmental Microbiology*, *72*(3), 2064–2069.

Charernsriwilaiwat, N., Rojanarata, T., Ngwhirunpat, T., Sukma, M., & Opanasopit, P. (2013). Electrospun chitosan-based nanofiber mats loaded with *Garcinia mangostana* extracts. *International Journal of Pharmaceutics*, *452*(1-2), 333–343.

El-Sharif, A. A., & Hussain, M. H. (2011). Chitosan–EDTA new combination is a promising candidate for treatment of bacterial and fungal infections. *Current Microbiology*, *62*, 739–745.

Frandsen, E. V., Pedrazzoli, V., & Kilian, M. (1991). Ecology of viridians *streptococci* in the oral cavity and pharynx. *Oral Microbiology and Immunology*, *6*, 129–133.

Greenberg, M., Dodds, M., & Tian, M. (2008). Naturally occurring phenolic antibacterial compounds show effectiveness against oral bacteria by a quantitative structure–activity relationship study. *Journal of Agricultural Food Chemistry*, *56*(23), 11151–11156.

Han, B., Wei, Y., Jia, X., Xu, J., & Li, G. (2012). Correlation of the structure, properties, and antimicrobial activity of a soluble thiolated chitosan derivative. *Journal of Applied Polymer Science*, *125*(S2), E143–E148.

Hindler, J. A. (2000). Special antimicrobial susceptibility test. In C. R. Mahon, & G. Manuselis (Eds.), *Textbook of the diagnostic microbiology* (pp. 97–104). Philadelphia: WB Saunders.

Hotta, M., Nakajima, H., Yamamoto, K., & Aono, M. (1998). Antibacterial temporary filling materials: The effect of adding various ratios of Ag–Zn–Zeolite. *Journal of Oral Rehabilitation*, *23*, 485–489.

Jorgensen, J. H., & Turnidge, J. D. (2007). Antibacterial susceptibility tests: Dilution and disk diffusion methods. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, & M. A. Pfaller (Eds.), *Manual of clinical microbiology* (pp. 1152–1172). Washington DC: American Society for Microbiology.

Koh, J. J., Qiu, S., Zou, H., Lakshminarayanan, R., Li, J., Zhou, X., et al. (2013). Rapid bactericidal action of alpha-mangostin against MRSA as an outcome of membrane targeting. *Biochimica et Biophysica Acta (BBA)—Biomembranes*, *1828*(2), 834–844.

Langoth, N., Kalbe, J., & Bernkop-Schnürch, A. (2003). Development of buccal drug delivery systems based on a thiolated polymer. *International Journal of Pharmaceutics*, *252*, 141–148.

Li, B., & Robinson, J. R. (2005). Preclinical assessment of oral mucosal drug delivery systems. In T. K. Ghosh, & W. R. Pfister (Eds.), *Drug delivery to the oral cavity: Molecules to market* (pp. 41–66). Florida: CRC Press.

Marschütz, M. K., & Bernkop-Schnürch, A. (2002). Thiolated polymers: Self-crosslinking properties of thiolated 450 kDa poly(acrylic acid) and their influence on mucoadhesion. *European Journal of Pharmaceutical Sciences*, *15*(4), 387–394.

Morales, J. O., & McConville, J. T. (2011). Manufacture and characterization of mucoadhesive buccal films. *European Journal of Pharmaceutical and Biopharmaceutics*, *77*(2), 187–199.

Mueller, C., Verroken, A., Iqbal, J., & Bernkop-Schnürch, A. (2013). Thiolated chitosans: In vitro comparison of mucoadhesive properties. *Journal of Applied Polymer Science*, *124*(6), 5046–5055.

Nakagawa, Y., Iinuma, M., Naoe, T., Nozawa, Y., & Akao, Y. (2007). Characterized mechanism of α-mangostin-induced cell death: Caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells. *Bioorganic and Medicinal Chemistry*, *15*(16), 5620–5628.

Pedraza-Chaverri, J., Cárdenas-Rodríguez, N., Orozco-Ibarra, M., & Pérez-Rojas, J. M. (2008). Medicinal properties of mangosteen (*Garcinia mangostana*). *Food and Chemical Toxicology*, *46*(10), 3227–3239.

Pothitirat, W., Chomnawang, M. T., Supabphol, R., & Gritsanapan, W. (2009). Comparison of bioactive compounds content, free radical scavenging and anti-acne inducing bacteria activities of extracts from the mangosteen fruit rind at two stages of maturity. *Fitoterapia*, *80*(7), 442-427

Silber, M. L., & Fellman, J. K. (2006). A new tannin bioassay protocol uses a highly stable, hydrophobically associated protein-dye marker that allows rapid, accurate and low-cost tannin monitoring. *Wine Business Monthly*, *13*(2), 64–70.

Sill, T. J., & Recum, H. A. (2008). Electrospinning: Applications in drug delivery and tissue engineering. *Biomaterials*, *29*, 1989–2006.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Method in Enzymology*, *299*, 152–178.

Sudhakar, Y., Kuotsu, K., & Bandyopadhyay, A. K. (2006). Buccal bioadhesive drug delivery—A promising option for orally less efficient drugs. *Journal of Controlled Release*, *114*(1), 15–40.

Thirawong, N., Nunthanid, J., Puttipipatkhachorn, S., & Sriamornsak, P. (2007). Mucoadhesive properties of various pectins on gastrointestinal mucosa: An in vitro evaluation using texture analyzer. *European Journal of Pharmaceutics and Biopharmaceutics*, *67*(1), 132–140.

White, R. L., Burgess, D. S., Manduru, M., & Bosso, J. A. (1996). Comparison of three different in vitro methods of detecting synergy: Time-kill, checkerboard, and E test. *Antimicrobial Agents and Chemotherapy*, *40*(8), 1914–1918.

Yichu, S., & Zhang, W. (2010). Preparative separation of major xanthones from mangosteen pericarp using high-performance centrifugal partition chromatography. *Journal of Separation Science*, *33*(9), 1274–1278.

Zong, X., Kim, K., Fang, D., Ran, S., Hsiao, B. S., & Chu, B. (2002). Structure and process relationship of electrospun bioabsorbable nanofiber membranes. *Polymer*, *43*, 4403–4412.