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Gellan Gum Film Containing Virgin Coconut Oil for Wound Dressing Application

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Abstract. Virgin coconut oil (VCO) is a promising candidate in promoting healing process due to its biocompatibility and antibacterial activities. In this work, we investigated the potential of VCO incorporated in gellan gum (GG) films to apply as wound dressing materials. Non-ionic surfactants, i.e., Triton X-102 and Tween-20 were used to obtain stable emulsions due to immiscible behavior between GG and VCO. Stable formation of GG-VCO films was successfully obtained by using surfactants at above their critical micelle concentrations, i.e. 1% (w/v) for Triton X-102 and 1.2% (w/v) for Tween-20. Cell proliferation exhibited that VCO is non-cytotoxic to human skin fibroblast cells (CRL2522) with limited cell growth observed on the film's surface which might be due to hydrophobic influence of physic-chemistry of the materials surface. The *in-vitro* qualitative bacterial study showed that GG-VCO films do not inhibit both *Escherichia coli* and *Staphylococcus aureus* at all concentrations.

Keywords: gellan, virgin coconut oil, cell studies, antibacterial

1. Introduction

The development of wound care materials continues to cater to the various different needs of damaged skin problems. Biopolymers such as gellan gum (GG), produced by Pseudomonas elodea have received great attention, particularly in the field of biomedicine, due to their biocompatibility and biodegradability properties. Several studies have been conducted by using GG on for medical and pharmaceutical applications, such as dual layer films, dressing material, scaffold and as a vehicle for ophthalmic drugs. Besides that, it demonstrates good compatibility with live cells such as mouse fibroblast (L929 cell line), human dermal fibroblasts (HDFs), human fetal osteoblasts (hFOBs 1.19), human nasal cartilage, and rat bone marrow cells (rBMC).

On the other hand, virgin coconut oil (VCO) which is extracted from fresh coconut meat under mild temperatures is a promising candidate in promoting healing process due to its biocompatibility and antibacterial activities. It contains Lauric acid (C12) as the major component of fatty acid, reported at 78%, and followed by myristic acid (C14) at 8% [1]. A few studies reported that pure VCO examined through in-vivo test promotes the healing process [2]-[4]. In combating bacteria, Shiling and co-workers successfully demonstrated the growth inhibition of Clostridium difficile by fatty acids of VCO [5]. However, to our knowledge no study has been conducted to examine the biocompatibility of VCO composite films through in-vitro tests, specifically in assessing the potential of VCO to be used as wound dressing materials. In this study, we describe the preparation of the GG-VCO films and their effect on human cell lines as well as bacteria. Surfactants were added into the GG-VCO emulsions to produce a smooth-wrinkle free films. The in-vitro test involved cell proliferation which was quantified on human skin fibroblast cells (CRL-2522, American Type Tissue Collection). Meanwhile, the antibacterial studies (qualitative analysis) of GG-VCO

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composite films were assessed on Gram-positive (Staphylococcus aureus) and Gram-negative bacteria (Escherichia coli) through their inhibition zone.

2. Experimental

2.1. Materials and Film Formation

Low-acyl gellan gum (Gelzan™ CM, $M_w \approx$ 2-3 x 10^5 Da, product number-G1910, lot number SLBB0374V), Triton™ X-102 (product number-1001318460, lot number MKBDD4707V) and Tween®20 (product number-1379, lot number 032K0182,) were obtained from Sigma-Aldrich, Malaysia. Virgin coconut oil (VCO, product number-617488-D) was obtained from BioNeutraceutical (M) Sdn. Bhd, and norfloxacin (product number-N9890) was from Fluka, Malaysia. All materials were used as initially received.

The films were prepared by using the casting method. A stock solution of gellan gum (GG) was prepared by dissolving 1% (w/v) of GG in 100 mL deionized water (18.2 MΩ) with continuous stirring for 2 h at 70 ℃. Virgin coconut oil (VCO) solutions at concentrations of 0.3% (w/w), containing non-ionic surfactants of Triton-X or Tween 20 (0.1-1.2% w/v) were stirred at 500 rpm until the solution became clear. Control GG-VCO films without surfactants were prepared using a high-speed homogenizer (Ultra-Turrax T25, IKA-Works, Inc., USA) and homogenized at 20,000 rpm for 2 min to obtain a stable emulsion. GG solutions and VCO containing non-ionic surfactants solutions were then stirred at 500 rpm for 2 h at 70 ℃ to obtain a stable emulsion. Emulsions of GG-VCO (0.3% w/v) with and without surfactants were stable even after 4 weeks with no phase separation was observed (Fig. 1[a]). The emulsions of GG-VCO were then deposited onto petri dishes (90 mm x 15 mm) and placed inside a Venticell oven at 30 ℃ for at least 24 h. Control films of gellan gum-norfloxacin were prepared by adding 0.01% (w/w) of norfloxacin into a stock gellan gum solution. All films were pre-conditioning in a desiccator (24 ℃, 50 % relative humidity (RH)) for at least 2 days prior to any testing. The transmittance of the films were recorded on a Varian Cary 50 spectrophotometer at 24 ℃ with data interval = 0.5 nm and a scan speed = 300 nm/min.

2.2. Cell studies

The cultivation of normal human skin fibroblast cells (CRL2522-ATCC) in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10 % (v/v) fetal bovine serum (Sigma Aldrich, Malaysia) and 1% (v/v) antibiotic (Penicillin/ Streptomycin, Sciencell, USA) were used as the cultured medium. Cells were stored at 37 ℃ in a humidified 5 % CO_2 atmosphere and were sub-cultured every 3 days per established protocols, then harvested at 60-80 % confluence.

Film samples were soaked with 70 % alcohol for 5 min before placing into 96-wells plate, which then sterilized in a biological safety cabinet under UV radiation for 20 min. Prior to cell seeding, samples were soaked in culture media (EMEM only) for 24 h and the supernatant were removed before CRL2522 cells (5000 cells/ well) were seeded into wells containing film samples and incubated at 37 ℃ in a humidified atmosphere of 5 % CO_2 atmosphere for 24, 48 and 72 h. Tissue culture polystyrene plates (TCPP) were used as control. Cell proliferations were quantified by using ~~a~~ CellTiter 96 ® aqueous one solution assay (Promega, USA) containing a tetrazolium compound [3]-[5][-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salts; MTS(a)] and an electron-coupling reagent (phenazineethosulfate). Prior to addition of the solution assay (20 µL in each wells), the media in all wells containing samples, except for control, were replaced with fresh media and later incubated for 3 h in the same condition. Then, 100 µL of inoculants were transferred into new wells and the absorbance was measured by using a microplate reader (Multiskan Ascent 96/384, USA) at 490 nm. The absorbance converted to cell number using calibration curves of CRL2522 cells in 96-well plates under the same condition.

2.3. Bacterial studies

Gram-positive (*Staphylococcus aures* - *S. aures*) and Gram-negative (*Escherichia coli* – *E. coli*) were used for anti-bacterial assays. Mueller-Hinton (MH) standard growth medium (Difco, Malaysia) were sterilized by autoclave for 15 min at 120 ℃. *S. aures* and *E.coli* were grown in MH agar and incubated aerobically at 37 ℃ for 24 h in an incubator to obtain suspensions of 0.5 optical densities at 600 nm

(Biomerieux Densicheck Plus). Inoculants of both *S. aures* and *E. coli* were evenly spread on the solid MH agar using swab sticks and dried before inserting the samples discs (d ~ 6 mm, three replicates). The plates were examined for the presence of growth inhibition zone after the incubation.

3. Results and Discussion

3.1. Effect of surfactant

In general, gellan gum solution is immiscible with virgin coconut oil due to its high interfacial tension, which is typical for oils with most polymers. The interfacial tension was reduced by introducing the surfactants which allowed the emulsion polymerization process took place. In this study, non-ionic surfactants, i.e. Triton X-102 and Tween-20 were used in preparing the gellan gum-virgin coconut oil (GG-VCO) films. Free-standing GG and GG-VCO films with no surfactant were prepared as the controls. GG-VCO films with no surfactant, homogenized at 20,000 rpm produced dwindle-shape films (Fig. 1[c]), which opposed to a perfect shape of gellan gum film (Fig. 1[b]). This could be due to fewer molecules of VCO as a non-polar substance solubilized in GG solution.

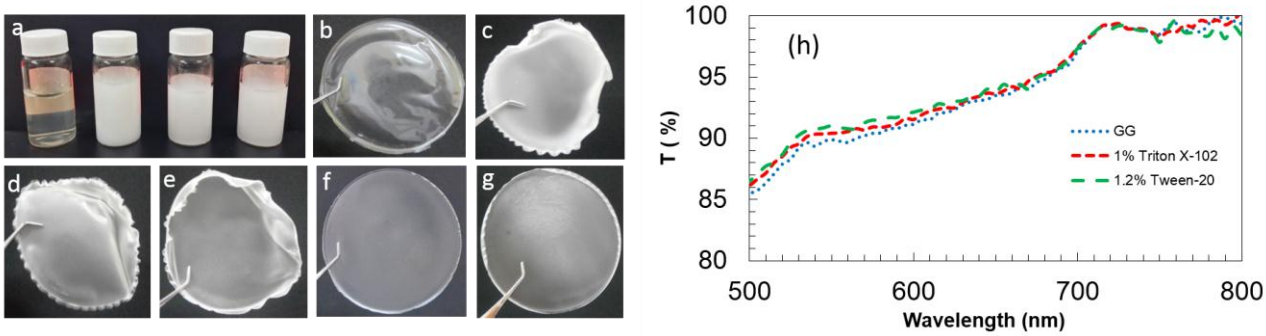

Fig. 1: (a) Stability of the GG-VCO emulsions (from left to right-control (GG), VCO with no surfactant, 1% w/v Triton X-102, and 1.2% w/v Tween-20), the appearance of (b) GG films, (c) GG-VCO film with no surfactant, (d) GG-VCO film with 0.5% w/v Triton X-102, (e) GG-VCO film with 0.8% w/v Tween-20, (f-g) GG-VCO films containing Triton X-102 and Tween-20 at 1.0% and 1.2% w/v, respectively, and (h) transmittance of GG film and GG-VCO with 1% w/v Triton X-102 and 1.2% w/v Tween-20 films.

The addition of Triton X-102 and Tween-20 of non-ionic surfactants in GG-VCO emulsions assisted in improving the physical formation of the GG-VCO films at above their critical micelle concentration (CMC). Below the CMC point, the phase separation was observed on the GG-VCO films with Triton X-102 (0.5% w/v) and Tween-20 (0.8% w/v) (Fig. 1[d-e]) which were quite similar to the GG-VCO film with no surfactant (Fig. 1[c]). The GG-VCO composite films were transformed to a smooth wrinkle-free at concentration of 1% (w/v) for Triton X-102, and 1.2% (w/v) for Tween-20 (Figure 1[f and g]). At these particular concentrations, the surfactant form sufficient micelles, which were capable of solubilizing the non-polar substances of VCO and interacting with GG. The GG-VCO films containing Triton X-102 and Tween-20 at 1.0% and 1.2% (w/v) respectively were opted for cell proliferation studies. All GG-VCO films with Triton X-102 and Tween-20 were transparent which show identical transmittances around ≈ 95% (λ = 700 nm), as to free-standing GG film (Fig.1 [h]).

3.2. Cell proliferation

The cell proliferation's quantification by using CellTiter 96 ® aqueous one solution assay of GG-VCO films containing Triton X-102 and Tween-20 surfactants on human skin fibroblast cells (CRL2522) is shown in Fig. 2. Compared to GG film, GG-VCO film with Triton X-102 recorded almost an identical number of cells after incubated for 24 h, i.e., at 3,000 cells/well and GG film at 2670 cells/well. On the other hand, GG-VCO film with Tween-20 recorded the lowest cell number at 830 cells/well at the same duration. However, the cell growth on GG films increased significantly after being incubated for 48 h where the increment recorded for ≈ 5-fold than 24 h to 14,000 cells/well. The growth on GG-VCO film with Triton X-102 was maintained at 2,850 cells/well and the film with Tween-20 increased almost ≈ 4-fold to 3,300 cells/well. Even though the increment of cell growth on GG-VCO film with Tween-20 is quite significant, but the

number is in the same range as recorded on GG-VCO with Triton X-102 film. After 72 h of incubation, the GG film again clearly shows a solid growth to 23,100 cells/well which supports the biocompatibility of GG on CRL2522 cells, that is an in agreement with other previous studies on various cells lines [6]. However, the cell growth on GG-VCO films containing Triton X-102 and Tween-20 decreased to ≈ 1-fold and 3-fold after 72 h of incubation, respectively compared at 48 h.

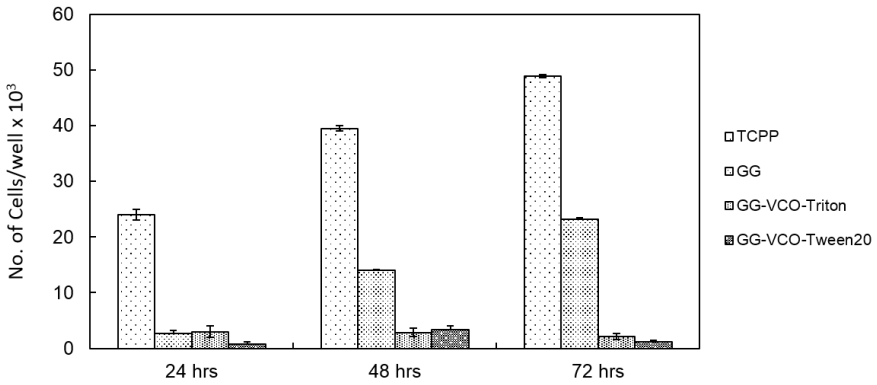

Fig. 2: Cell proliferation of GG and GG-VCO containing Triton X-102 and Tween-20 films cultured in the medium containing human skin fibroblast cells (CRL2522) for 72 h. Error bars indicated standard deviation (n=3).

The low number of CRL2522 cells attached on the GG-VCO films containing Triton X-102 and Tween-20 could be due to the influence of physic-chemistry of the material's surface. A few studies have been reported the effect of surface roughness, hardness, hydrophobicity and hydrophilicity of the materials to the biological response of viable cells [7]-[10]. In this study, the use of VCO which is a hydrophobic material in the GG film could possibly limit the adherence of CRL2522 cells on the composite film. Furthermore, the micelles' formation on the surface of composite films due to emulsion polymerization process (result not shown) could be another factor which distorted the cell growth. Further study will be carried out in order to understand the effect of VCO to cell growth in composite film.

3.3. Antibacterial activity

Antibacterial activities of GG-VCO containing Triton X-102 composite films were studied through qualitative method against Gram-negative (Escherichia coli - *E. coli*) and Gram-positive (Staphylococcus aureus - *S. aureus*) bacteria (Fig. 3). The positive control sample, i.e. gellan gum with 0.01% (w/w) of norfloxacin clearly shows the inhibition zones (≈ 18 mm) on both *S. aureus* and *E. coli* bacteria. The addition of VCO at 0.3% w/v in GG film however resulted with no clear zone of inhibition around the samples against both bacteria. It has been elaborated that the VCO does not possess antibacterial activity by its own, but rather induced by its free fatty acids, particularly lauric acid (C12), and small extent of capric acid (C10) and caprylic acid (C8) [5]. In another word, the VCO must be metabolized to release those components and exert its antimicrobial effects [11]. The derivative of monolyceride, known as monolaurin or monoester of the lauric acid is the most effective in inhibiting the microorganism by disrupting the cell membrane and the cytoplasm of cells [5].

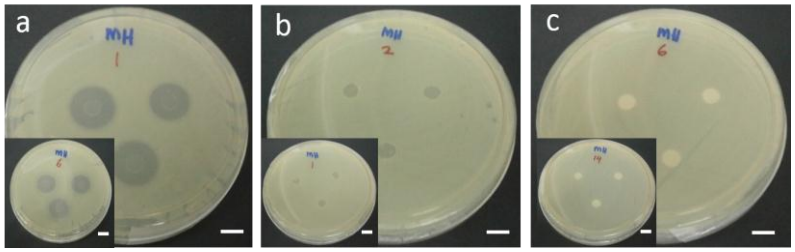

Fig. 3: Qualitative results of (a) GG-norfloxacin, (b) GG and (c) GG-VCO containing Triton X-102 films against *Escherichia coli*. Insert: The films against *Staphylococcus aureus.* Scale bars represent 1 cm.

In this particular study, the negative observation of GG-VCO films with Triton X-102 against *E. coli* and *S. aureus* could be due to lesser degree of VCO has contact with MH agar, which reduced the chances of the

latter to diffuse into the agar. As mentioned earlier, the antibacterial activities of VCO is triggered by the monolaurin of lactic acid, which off-course dependent to the concentration of VCO. Higher contents of VCO (> 0.3% w/v) may have higher content of monoglyceride to transform to monolaurin derivative and thus exert the antimicrobial effects. This assumption is supported by a study conducted by Altiok and co-workers in their research in the examination of antibacterial properties of chitosan film with tyme oil [13]. In their study, chitosan film with thyme oil at concentrations of 0.2%, 0.4% and 0.6% (v/v) showed no inhibition zones on all 4 bacteria tested, i.e. *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. However, the inhibition zones were observed on the chitosan film with higher concentrations of thyme oil ranging 0.8% - 1.2% (v/v) with clear zones recorded at 15.5 – 19 mm [13]. The authors proposed the killing of the bacteria at higher concentrations of thyme oil is due to the hydrophobic effect of phenolic groups, namely carvacrol and thymol which cause deterioration of the bacteria membrane, thus the bacteria died. Further study should be carried out to understand the effect of VCO at high concentrations in gellan gum materials against micro-organism.

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