Research Article

Development of Lion's Mane Mushroom Extract-Loaded Polyvinyl Alcohol/Chitosan Hydrogel Film Composites for Controlled Release of Ergosterol

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Abstract
Ergosterol is the most prevalent sterol in microbial membranes, which shows a hypoglycemic effect that can be used to treat type 2 diabetes. However, ergosterol has restricted bioavailability and low free-form solubility in hydrophilic conditions. This research aimed to evaluate the properties of bioactive compounds, ergosterol, from Lion's Mane mushroom (LM) mushroom extract and entrap them in polyvinyl alcohol (PVA) and chitosan (CS) hydrogel film composites with/without the crosslinker tetraethoxysilane (TEOS) to increase the solubility of ergosterol. Ergosterol-enriched extract (37.17 mg/g extract) was extracted from the LM by supercritical fluid extraction using CO_2 (SCFE-CO_2). LM extract showed no cytotoxic effects on the fibroblast cells, with cell viability ranging from 94.60–97.40%, increased cell proliferation, and wound-healing activity at 1 mg/mL of LM extract. Scanning Electron Microscopy revealed that the film exhibited a homogenous structure with swelling ability. The release rate of ergosterol from the film was relatively higher during 30 to 60 min of submersion in phosphate buffer. LM extract-loaded PVA/CS hydrogel film composites have a high potential for wound-healing applications in medical settings. This research challenges further investigation for alternative treatments and offering aids for efficiency improvement in wound-healing. The longer release rate of LM extracts should be investigated in future work.

Keywords: Chitosan, Ergosterol, Hydrogel, Polyvinyl alcohol, Tetraethoxysilane

1 Introduction

Drug delivery systems can facilitate the delivery of active pharmaceutical compounds to enhance the desired therapeutic effect. There are numerous problems in the traditional drug delivery mechanisms, such as toxicity and repetitive treatment. The creation of controlled drug delivery systems can resolve the issues in conventional pharmaceutical administration [1]. The application of hydrogels could be a choice to maximize therapeutic advantages. Hydrogels are made from a polymer matrix that can hold a significant volume of aqueous media. Hydrogels have several uses, such as wound treatment, ophthalmic systems, and scaffolds for bone and tissue engineering [2]. In the biomedical area, hydrogels have been used as materials for tissue-designed scaffolds, biosensors for microfluidics, actuators for optics, standard extracellular matrices for biological investigation, and vehicles for drug delivery systems [3]. Drug delivery

systems using hydrogels decrease the dosage needed, maintain the desired therapeutic concentration in a single administration, and lessen adverse effects by preventing drug accumulation in the tissues. In addition, a hydrogel can prolong the release of pharmaceuticals by simple preparation [4]. There is intensive research on natural polymers that resemble physiological tissues. Hydrogel-based drug delivery methods employ natural polymers such as starch, chitosan (CS), collagen, gelatin, zein, and albumin for various biomedical applications [3], [5]–[7].

CS was regarded as a biopolymer prepared from natural resources. It is a linear polysaccharide consisting of glucosamine and N-acetyl glucosamine units. Chitin from crustaceans, such as shrimp shells, is soaked in an alkaline solution to deacetylate the chitin in the shells [8]. Moreover, chitin from other sources, not related to crustaceans, is also manufactured using alternative processes. For various biological uses, including the delivery of drugs, CS has been extensively synthesized into various forms, including fibers, films, and especially hydrogels. Non-covalent interactions between polymer chains, such as hydrogen bonding, hydrophobicity, and electrostatic forces, produce physical hydrogels made from CS [9]. CS is non-antigenic, indicating several benefits, including low toxicity, excellent biocompatibility, and support for cell adhesion and proliferation [8]. Hydrogels derived from poly (vinyl alcohol), or PVA, are well-characterized matrices used in biomedical and clinical settings for decades [10]. PVA was employed to make up the characteristics of CS and control the drug release from the hydrogel [2]. PVA combined with CS was used as wall material to encapsulate natural extracts for antibacterial wound-healing. PVA/CS membranes with bioactive compounds from the root extracts of *Calotropis procera* [11] and PVA/CS hydrogels containing silver nanoparticles and the extract of *Brassica oleracea* [12] could lessen pain frequency and promote a higher wound-healing efficiency. The network formation of covalent cross-linking in the hydrogel may be established by using tetraethoxysilane (TEOS) [3]. Using TEOS as a cross-linker in PVA/CS hydrogel film composite revealed improved interfacial adhesion and film mechanical characteristics of the composite materials [13].

LM, *Hericium erinaceus*, is a medicinal mushroom containing several bioactive compounds used for food supplements and medicinal products [14], [15]. *Hericium* contains several unique bioactive compounds, including erinacines and hericenones. These compounds exhibit beneficial effects on human health, such as neuroprotection, anti-cancer, and anti-inflammation [15]. LM presents a dominant content of hericenone C, hericene A, and especially ergosterol [16]–[18]. Gasecka *et al*., [19] reported that more than 4.5 mg of ergosterol/g dry weight was found in the fruiting bodies of LM. Ergosterol and its peroxidation products have strong bioactivity exhibiting antioxidant, anti-inflammatory, and antitumor properties [20]. Anti-inflammatory properties of ergosterol are associated with inhibiting pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β [21]. Despite having a high ergosterol content, the bioavailability of ergosterol is restricted because it has low free-form solubility in both lipophilic and hydrophilic conditions [22]. Consequently, the solubility of the bioactive compound in LM should be improved for medicinal applications or nutritional supplements. It was discovered that the PVA/CS-hydrogel composites produced by a traditional solvent-casting technique could encapsulate the phenolic compounds, which have potent antioxidant properties and excellent drug-delivery properties [8]. This is an interesting approach to improve the solubility of bioactive compounds in the LM.

This study used the green process, supercritical fluid extraction using CO_2 (SCFE-CO_2), to extract LM. According to our previous study, the optimal SCFE-CO_2 processing conditions were employed to maximize the extraction yield, ergosterol, and phenolic contents with the highest antioxidant activity [23]. This research aimed to entrap LM extract obtained by SCFE-CO_2 in the hydrogel film composites of PVA and CS prepared by the solvent-casting method with/without cross-linking agent TEOS. Encapsulation was performed to capture the substances within the LM extract and solubilize them in the buffer solution more efficiently. The mechanism involves inducing the inter- and intra-molecular hydrogen bonds between PVA and CS solutions with TEOS. LM extract was integrated into the cavity of the polymer solution through hydrogen bond formation. The Si-O bonds of TEOS lead to interfacial adhesion of composite materials and form LM extract loaded-PVA/CS hydrogel film composites. LM extract from SCFE-CO_2 was evaluated for its medicinal properties, such as enhancing fibroblast proliferation and migration efficiency. Some properties of the composite hydrogel film, such as swelling behavior and the release characteristics of ergosterol from the film, were performed to evaluate their potential for medicinal applications. In addition, the morphology and chemical bonding inside the composite hydrogel

film were also investigated. This study demonstrated the production of composite hydrogel films from PVA, CS, and TEOS for loading bioactive compounds in LM extract. These films can potentially be used in medical applications as wound-healing hydrogel. The loading of LM extract into hydrogel film composites led to an increased solubility of ergosterol in the phosphate buffer, which broadened their application for medical purposes.

2 Materials and Methods

2.1 *Materials*

Irregular-shaped fruiting bodies of LM were obtained from Fresh and Friendly Farm Co., Ltd. (Pathum Thani, Thailand). Carbon dioxide gas was bought from the Bombay Carbon Dioxide Gas Company (Mumbai, India). Absolute ethanol was obtained from PanReac (Barcelona, Spain). Water (HPLC grade) and methanol (HPLC grade) were purchased from LAB-SCAN (Gliwice, Polland). Ergosterol, 95% in purity, was bought from Sigma-Aldrich, Co., Ltd. (Massachusetts, United States). Chitosan powder (90.0% degree of deacetylation, intermediate molecular weight, 200,000 Da) was purchased from Seafresh Chitosan (LAB), Co., Ltd. (Chumphon, Thailand). Polyvinyl alcohol with a molecular weight of 15,000 g/mol and tetraethyl orthosilicate (TEOS) were obtained from Sigma Aldrich Co., Ltd. (Massachusetts, United States), while acetic acid was from QReC Co., Ltd. (New Zealand).

Figure 1: LM extract obtained by supercritical fluid extraction using carbon dioxide (SCFE-CO_2).

2.2 *Preparation of supercritical CO_2 LM extract*

Supercritical fluid extraction using carbon dioxide (SCFE-CO_2) was used for extracting LM. The extraction was carried out, following the previous study [23], at 36.8 °C, 100 bar with Applied Separations, supercritical equipment for a laboratory-scale (Allentown, PA, USA) with EtOH as a co-solvent at 0.99 mL/min. The CO_2 flow rate was fixed at 3 L/min for 60 min. The LM sample vessel was filled with polypropylene wool and then put in the oven. A rotary evaporator (R114, Buchi, Switzerland) at 50 °C was used to evaporate the EtOH in the extract for about 15 min, collected and stored at –20 °C until further investigation. The LM extract by SCFE-CO_2 is presented in Figure 1.

2.3 *Quantification of ergosterol*

Determination of ergosterol in the LM extract was performed with the previous method [14] using high-performance liquid chromatography (HPLC), Agilent Technologies 1200 series, Germany. The HPLC system, which had an Eclipse Zorbax XDB-C 18 (Agilent, 250 x 4.6 mm, 5 µm) analytical column, was linked to the UV-VIS LC detector. The used mobile phase were methanol and water at a 98:2 ratio by volume. The LM extract was detected with an absorbance of 282 nm, calculated from the ergosterol standard calibration curve and exhibited as mg/g extract.

2.4 *Cell viability, cell proliferation, and wound-healing (scratch assay)*

Normal human dermal fibroblasts (HDF) were grown in the culture mixture of 10% (v/v) fetal bovine serum (FBS), streptomycin, and penicillin under standard conditions. Cells were used when it came to the fifteenth passage (a record of the number of times the culture has been sub-cultured). A temperature- and humidity-controlled incubator (2123TC, Cornelius, USA) was used for cell cultivation at 37 °C [24].

For cell viability and cell proliferation assay, the 96-well plates were filled with 1×10^5 cells per well of the HDF, which were then incubated at 37 °C with 5% CO_2 throughout the night to promote cell adhesion. Five concentrations of the LM extracts (0.001–1 mg/mL) were exposed to the cells for 24 h. Sodium lauryl sulfate (0.001–1 mg/mL) served as a negative control for cell survival. The adherent cells were subsequently washed, stained with Sulforhodamine B (SRB), and fixed after the incubation. The absorbance at 510 nm was determined using a multi-well microplate reader (Wallac Victor V, PerkinElmer). Cell viability was calculated compared with the control [25], while cell proliferation was determined using the equation of Manosroi *et al*., [24]. As a positive control, ascorbic acid (0.001–1 mg/mL) was used. At

540 nm, the absorbance of the sample solution was measured after the dissolution of the bound dye.

Wound-healing (Scratch assay) was performed [26]. HDF cells were placed at 3×10^5 cells/mL in a 24-well plate containing 5% FBS and expanded to form a confluent cell monolayer. A 200 µL pipette tip scratched a small area of the cell monolayer. To get rid of cell debris, the cells were washed in phosphate-buffered saline (PBS). After replenishing the plates with fibroblast medium containing various SCFE-CO_2 LM extracts at a dose of 1 mg/mL, the plates were incubated at 37 °C with 5% CO_2. Ascorbic acid was used as the positive control. After scratching with a pipette tip for 0, 6, 24, and 48 h, the space between two cell layers was measured and examined under a microscope. The migration of HDF cells to cover the scratched area was recorded using a digital camera that could link to a microscope and computer system.

2.5 *Preparation of PVA and CS hydrogel film composites*

A wet conventional synthesis technique was used to prepare a PVA/CS hydrogel film composites. CS was dissolved in 3% v/v acetic acid and continuously stirred for 2 h at room temperature. PVA was also dissolved in 10% w/v DI water and stirred continuously for an hour at 80 °C. Then, the CS solution was poured into the PVA solution (PVA: CS at 1:1 by volume). The LM extract was loaded into the PVA/CS hydrogel film composites at 1 mg/mL. To create a homogenous gel, the mixture was stirred for 20 min at 50 °C. The gel was then placed on a petri dish, left for 24 h until it could be peeled from the dish, and dried at 40 °C in an oven overnight. The composite hydrogel film without TEOS was coded as PVA, PVA/CS, PVA/LM extract, and PVA/CS/LM extract. The mixture was then supplemented with 2% w/w of TEOS. For 40 min, the mixture was stirred at 50 °C to create a uniform gel. After that, the gel was placed on a petri dish and dried at 40 °C in the oven. The composite hydrogel film with cross-linker TEOS was coded as PVA/TEOS, PVA/CS /TEOS, PVA/LM extract/TEOS, and PVA/CS/LM extract/TEOS. Each sample was cut into a square shape (1.5×1.5 cm^2) for further analysis.

2.6 *Materials physical property and chemical composition of LM extract-loaded PVA/CS hydrogel film composites*

2.6.1 Swelling properties

The hydrogel swelling and equilibrium data were determined using the gravimetric technique. The sample was submerged in DI water for 48 h. The samples were removed from the solution after being submerged, dried with filter paper to eliminate any remaining water, and then weighed. Three samples were collected. The swelling ratio (Q) was determined as following Equation (1):

$$Q\ (g/g) = (W_{wet} - W_c)/W_c \quad (1)$$

where W_{wet} is the weight of a swollen composite hydrogel film, and W_c is the initial weight of the dried composite hydrogel film after drying at 40 °C in an oven.

2.6.2 In vitro release study

The PVA/CS/TEOS hydrogel film composites loaded with LM extract was dissolved in 30 mL of ethanol for 3 h in order to measure the amount of ergosterol present in each sample. Then, 0.1 mL of the solution was collected to measure the ergosterol content and set as the control. The release characteristics of ergosterol from each composite hydrogel film were investigated using the total immersion method [13]. Every sample was collected at 30, 60, 120, 180, and 300 min after being immersed in 30 mL of phosphate buffer solution, which mimicked the condition of human skin at 37 °C. The media solution, 0.1 mL, was taken out and replaced with fresh media solution in an equal amount to keep the volume constant. Then, the releasing profile of ergosterol from each composite hydrogel film was recorded.

As described above, ergosterol was determined using high-performance liquid chromatography and HPLC (Agilent Technologies 1200 series, Germany). Methanol and water were mixed in the mobile phase in a 98:2 volume ratio. At 282 nm, the absorbances were measured. The amount of ergosterol in the collected samples and the control was determined using the calibration curve of the ergosterol standard. Three replicates of each measurement were performed.

2.6.3 Scanning electron microscope (SEM)

SEM photographs of the composite hydrogel film were recorded using scanning electron microscopy (Quanta 450, Oregon, United States) at an acceleration voltage of 15 kV. The specimens were gold-coated

before the SEM observation with a magnification of 20000x.

2.6.4 Color measurement

The colorimeter (ColorFlex EZ, Hunter Lab, USA) was used to measure the color of the hydrogel. L^* (lightness), a^* (red-green), and b^* (yellow-blue) were used to interpret the color value. A standard white plate (L = 94.5, a = –1.17, b = 1.12) was used for the instrument calibration. L^* represents the brightness index, L^*= 0 means black, L^* = 100 means white; a^* corresponds to red and green, $+a^*$ means red and $–a^*$ green; b^* stands for blue and yellow, $+b^*$ is for yellow and $–b^*$is for blue. Figure 9(e) displays the implications of general color values ΔE [25]. ΔE was calculated from the following Equation (2):

$$\Delta E = \sqrt{(L^* - L_c)^2 + (a^* - a_c)^2 + (b^* - b_c)^2} \qquad (2)$$

where L^*, a^*, and b^* are the color values of the composite hydrogel films, and L_c, a_c, and b_c are the color values of the control (PVA hydrogel film composite).

2.6.5 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were measured using a Fourier transform infrared spectrometer (Invenio S, Bruker, USA). The measurement was performed with a sample and background scan frequency of 32 times at room temperature, with a resolution of ± 4 cm^{-1} in the spectral region of 4000 to 400 cm^{-1}.

2.7 *Statistical analysis*

One-way analysis of variance (ANOVA) was used to evaluate the data using SPSS 11.6 for Windows (SPSS Inc.). The Scheffe test was used to examine the differences between each sample at the *p*-value < 0.05 significant level.

3 Results and Discussion

3.1 *Properties of SCFE-CO_2 LM extract*

Ergosterol-enriched extract (37.17 mg/g extract) was successfully extracted from the LM by supercritical fluid extraction using CO_2 (SCFE-CO_2). The LM extraction using SCFE-CO_2 (extraction conditions: 46.38 °C, 100 bar, 0.99 mL/min EtOH for 60 min) yielded 37.14 mg of ergosterol in 1 g of LM extract, similar to our previous study [23]. Yongxia *et al*., [27] reported that the ergosterol extract from *Monascus anka* at 400 μg/mL significantly reduced the ROS levels and human skin fibroblast cell damage and showed several bioactivities such as anti-lipid peroxidation, antioxidant capacity, ability to protect and repair the damaged cells from H_2O_2 treatment. Ergosterol can repair the damaged fibroblast cells and inhibit melanoma growth and metastasis (skin cancer).

LH-1 is a kind of ergosterol that is derived from the marine fungus *Pestalotiopsis* sp. It suppressed migration in melanoma cells (A375 and B16-F10 cells) by downregulating the expression levels of MMP-9 and inducing cell death through the mitochondrial apoptosis pathway [28]. Ergosterol was not usually added for medicinal application because of its poor aqueous solubility [29]. According to Dong, Iqbal, and Zhao [30], utilizing ergosterol-loaded nanostructured lipid carriers (NLCs) had a more potent inhibitory impact than using pure ergosterol on the proliferation of high-glucose-stimulated mesangial cells and the development of extracellular matrix (ECM). NLCs could improve the solubility and bioavailability of ergosterol [30].

Therefore, the medicinal properties of the LM extract, which contains ergosterol, were determined, including toxicity and efficiency in enhancing fibroblast proliferation and migration (wound-healing), were determined. Then, the LM extract was entrapped with PVA/CS hydrogel film composite to increase its bioavailability for medicinal applications. The cell viability in Figure 2(a) shows that the incubation of human dermal fibroblast cells with LM extract at 0.0001 and 1 mg/mL indicated no cytotoxic effects on the cells, showing the cell viability ranging from 94.60 to 97.40%. However, when the negative control (sodium lauryl sulfate at 0.1–1 mg/mL) was used, the cell viability was reduced to 10.84%–8.41%.

LM extract at concentrations with no cell toxicity (0.01 and 1 mg/mL) could enhance the proliferation of fibroblast cells, as shown in Figure 2(b). The highest cell proliferation at 33.05% was observed with the LM extract at 1 mg/mL, whereas 0.1 mg/mL of ascorbic acid (positive control) exhibited higher cell proliferation at 45%. However, ascorbic acid at 1 mg/mL exhibited no activity on fibroblast proliferation, showing a negative percentage by the SRB assay, as presented in Figure 2(b). Therefore, the ability to enhance cell migration for wound-healing between LM extract and ascorbic acid was also compared in this study. Ziemlewska *et al*., [31]

reported that the LM extract (0.1 to 1 mg/mL) showed no cytotoxic effects on fibroblast cells and could promote cell viability and proliferation, which can be potentially used for cosmetic products with a wide range of functionality. SRB assay was used to measure cell viability and proliferation. Determination of total protein content in the cells can indicate the cell numbers in culture after standardization to ensure a linear relationship between the total protein content and the cell numbers. SRB assay could be applied to fibroblasts during wound-healing [32]. In the process of wound-healing, fibroblasts migrate to the site of injury, proliferate, get settled into the primary extracellular matrix (fibronectin, fibrin, and type 1 collagen), and accelerate the synthesis of extracellular matrix proteins, primarily collagen types 1 and 3 [33].

The scratch assay in Figure 3 using LM extract at the non-cytotoxic effect on HDF cells (1 mg/mL) for 6 h treatment exhibited no significantly different HDF cell migration compared to DMSO, DMEM, and ascorbic acid treatment. However, the wound-healing effects of LM extract in the HDF cell line were more prominent than DMSO and DMEM at 24 and 48 h. LM extract- and ascorbic acid-treated cells had spread to cover the scratched area. There were noticeable differences in the effects of LM extract at 24 and 48 h on the ability to repair wounds on the HDF cell line. The LM extract was shown to be active in promoting cell migration within 24 h and could close the wound by 48 h. However, the HDF cell migration at the scratch line with the LM extract after 48 h of the treatment (approximately 76% scratch wound coverage) was faster than the negative control (10% v/v DMSO and DMEM, with approximately 48% and 62% scratch wound coverage, respectively) but slower than ascorbic acid (approximately 87% scratch wound coverage). Even though ascorbic acid (1 mg/mL) can enhance the migration of HDF cells (Figure 3), it cannot increase the proliferation of HDF cells like the LM extract when used at the same concentration. These results demonstrated the ability of LM extract to accelerate wound closure and enhance the appearance of wound architecture without having any cytotoxic effects in the fibroblast wound-healing model. A deficiency in chemotherapeutic effectiveness arises from the significant incorrect delivery of cytotoxic drugs due to chemoresistance [34]. Consequently, patients may have an alternative form of therapy that involves the use of natural products.

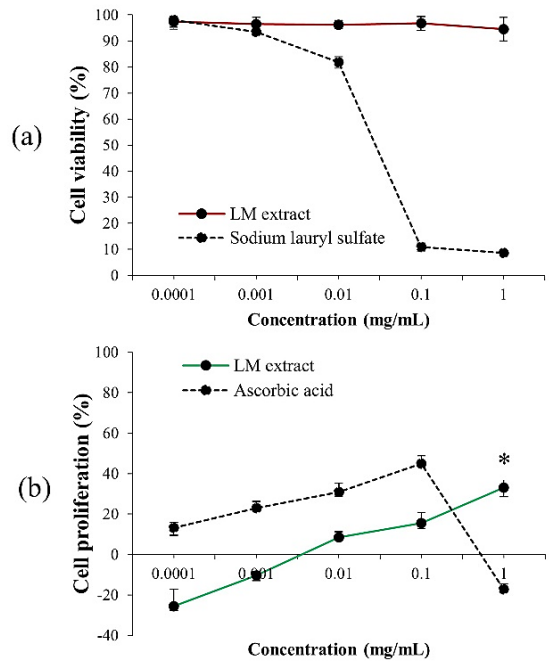

Figure 2: Cell viability (a) and cell proliferation (b) evaluation in fibroblast cell line after treatment with the Lion's Mane mushroom (LM) extract at different concentrations. Sodium lauryl sulfate served as a control for cell survival, and ascorbic acid was employed as a control for cell proliferation.

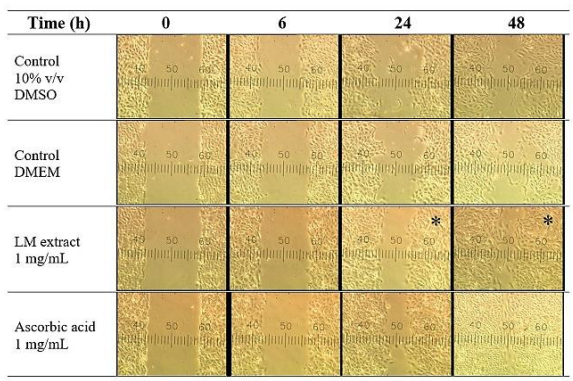

Figure 3: Evaluation of wound closure in a fibroblast cell line (40X) after treatment with dimethyl sulfoxide, DMSO (control), Dulbecco's Modified Eagle Medium, DMEM (control), Lion's Mane mushroom (LM) extract, and ascorbic acid, for 48 h.

A few studies have reported the possible impact of LM extract on wound-healing of fibroblasts. Abdulla *et al.*, [35] revealed that Sprague-Dawley rat wounds treated with an aqueous extract of LM (40 mg/mL)

significantly accelerated wound-healing. The histological investigation of the wound enclosure showed less scar width, decreased macrophage accumulation, and increased collagen and angiogenesis after the treatment with LM extract. The authors concluded that an aqueous LM extract significantly enhanced wound-healing in rats.

The LM in the form of aqueous and ethanolic extracts were used to treat the symptoms inside animal bodies, including gastric ulcers and inflammatory bowel disease (IBD). The LM aqueous extract significantly reduced the Sprague Dawley rats' ethanol-induced ulcers. With the use of the extract, the amount of ulcer areas in the gastric wall decreased, and antioxidant enzymes like catalase and superoxide dismutase were depleted. As shown by the MDA level, this reduced lipid peroxidation in the gastric tissue also inhibited edema and leucocyte infiltration of submucosal layers [36].

Ethanol extracts from LM were suggested by Qin *et al*., [37] as a preventive measure for IBD patients. According to their findings, oral administration of LM extract might significantly improve body weight and colon length (250 mg/kg/day and 500 mg/kg/day body weight). The mice given dextran sulfate sodium (DSS) had less intestinal bleeding. Nitric oxide, malondialdehyde, and superoxide dismutase production in serum may be adjusted to limit oxidative stress, and the LM extract can decrease the synthesis of inflammatory mediators in colon tissues, such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6.

In short, the wound-healing property of mushrooms in many studies was moderately associated with their rich content of polysaccharides from the aqueous extraction and bioactive components from both aqueous and methanol/ethanol extractions [38]. Primary and secondary triterpenes (0.156 mg/mL) from the *Ganoderma lucidum* methanol extract enhanced keratinocyte proliferation and migration without any toxicity symptoms or morphological effects [39]. In addition, the aqueous lyophilized *G. lucidum* extract promoted healing activity, demonstrated by enhanced collagen accumulation, total protein, hexosamine, and wound contraction in the experimental rats [40]. The Taiwanese Basidiomycete fungus *Antrodia camphorate* was discovered to have an aqueous extract that significantly accelerated wound-healing both *in vitro* by stimulating the proliferation of fibroblast cells and *in vivo* by reducing the number of inflammatory cells and increasing collagen synthesis in the injured tissues [41].

Consequently, LM extract had therapeutic effects on *in vitro* wound-healing; it was nontoxic to fibroblast cells at 1 mg/mL dosage, and cell viability ranged from 94.60–97.40%. LM extract could promote cell proliferation at wound scratching due to the HDF cell migration at the scratch line with the LM extract after 48 h of the treatment. The following study used SCFE-CO_2 LM extracts at a dose of 1 mg/mL to load the composite films.

3.2 *Physical property and chemical composition of LM extract-loaded PVA/CS hydrogel film composites*

3.2.1 Swelling ratio of hydrogel film composites

The swelling ability of composite hydrogel film is a crucial characteristic that demonstrates the potential for liquid absorption and drug delivery systems. The swelling ratio (swelling percentage) is related directly to the water quantity that composite hydrogel film can absorb. Hydrogels exhibit high water-absorbing capacity due to hydrophilic functional groups attached to their polymeric backbone. Hydrogels can also possess dissolving resistance because of the cross-linking within their network chains [42].

The swelling power of hydrogel film is attributed to the water-soluble polymers involved with two types of water molecules (bound water and bulk water) in the system. Bound water refers to the initial layer of water molecules that are firmly bonded to polymer chains. In comparison, bulk water, or free water, has substantially higher degrees of freedom than these water molecules [43]. PVA was the primary polymer used in hydrogel film composites in this study. The swelling ability of the hydrogel film composites with PVA might be due to the hydrogen bond interactions between water and the hydroxyl groups in the PVA structure. However, the hydrogen bonds are constantly breaking and reforming because PVA is easily degraded into water and carbon dioxide molecules during storage or due to microbiological activity [43], [44]. The PVA addition can improve the thermal stability and swelling ability of hydrogels [44].

The swelling ratio of hydrogel film composites as a function of time is presented in Figure 4. All composite hydrogel films without TEOS (Figure 4(a)) exhibited a consistent swelling ratio during 10 min, and some of the composite hydrogel films had completely dissolved. The combination PVA/CS/LM extract/TEOS hydrogel film composite showed the highest swelling percentage, up to 625%. All of the composite hydrogel film containing TEOS can absorb water over the 30 min test period (Figure 4(b)). The application of TEOS as a cross-linker for PVA/CS hydrogel film composites indicated enhanced film

mechanical properties and interfacial adhesion of composite materials because of the interaction between the amino groups of CS and Si-O bonds of TEOS [13]. This might explain why composite hydrogel film containing TEOS performed a higher consistency when immersed in water. Moreover, the swelling ratio was increased when CS was added to the composite hydrogel films (Figure 4(a) and (b)). A similar result was reported by Chopra *et al*. [45], who found that the swelling ratio increased with increased CS concentration. Chuysinuan *et al*., [46] described that the NH and OH groups of the CS-based hydrogel composites were deprotonated in water solution, resulting in an electrostatic repulsion along the CS and PVA chains in the hydrogel complex network, and swelling of the hydrogel.

Noticeably, the composite hydrogel films without TEOS in the combination of PVA/CS/LM extract exhibited a reduction in swelling ratio when compared with the combination of only PVA and CS in the hydrogel film (without LM extract), as shown in Figure 4(a). This result might also be related to the hydrophobicity of the LM extract that might prevent water from entering the gel 3D network. The swelling ratio was reduced from 318–380% to 238–296%, similar to the work reported by Liang *et al*. [47]. They found a significant reduction in swelling capacity when dihydrocaffeic acid (DA) and oxidized pullulan (OP) were added to the chitosan-based hydrogel film composites. DA and OP might disrupt the chain molecules' sequential arrangement and decrease the film's swelling capacity [43], [47]. The result differed from the hydrogel film composites obtained from the addition of TEOS (Figure 4(b)). The PVA/CS/LM extract hydrogel film composites showed a higher swelling ratio than PVA/CS hydrogel film composites without LM extract (Figure 4(b)). It seems that the differences in the swelling profile between PVA/CS/LM extract hydrogel film composites might be more related to the crosslinker TEOS, and the gel structure. TEOS was the key compound used in this research to improve the composite film's swelling capacity and consistency properties. The initial hydrolysis stages of TEOS occurred when water was added to TEOS. The water molecule could interact with the silicon atom, leading to hypervalent substrate formation [48], while all side chains of TEOS could still form bonds with PVA, CS, or LM extract. Therefore, adding TEOS to the hydrogel film composites increases swelling ability.

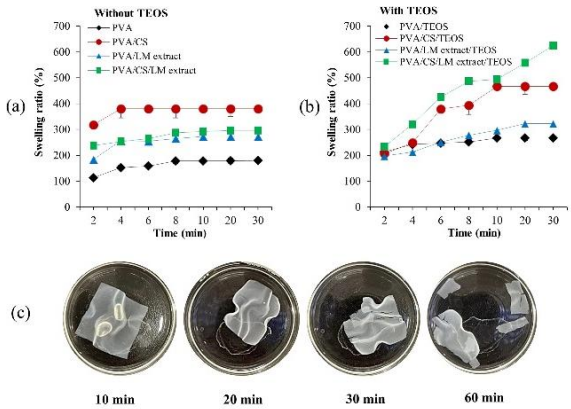

Figure 4: Swelling ratio of the composite hydrogel films prepared from different polymers without (a) and with (b) crosslinker tetraethoxysilane (TEOS) and Lion's Mane mushroom (LM) extract loading, and the appearance of PVA/CS/LM extract/TEOS at different soaking time after contacting with the dissecting needle to transfer the film (c).

Similar results were reported by Thanyacharoen *et al*., [13], who found that the swelling of the PVA/CS/TEOS hydrogel film composites was enhanced when black rice extract was added to the film. Phenolic compounds and the hydroxyl functional groups in the black rice extract might react with water or solution, thereby increasing the swelling of the hydrogel film composites [13]. The increased swelling of the PVA/CS/TEOS hydrogel film composites containing LM extract (Figure 4(b)) could be attributed to the functional groups of compounds inside the extract, including phenolic compounds and ergosterol, and the gel structure with TEOS. The presence of phenolic compounds and ergosterol in LM extract from SCFE-CO_2 was confirmed in our previous studies [16], [23]. The LM extract from SCFE-CO_2 at 70 °C for 350 bar contained a total phenolic content of up to 0.2 mg GAE/mg dried LM and 1.11 mg/g dried LM for ergosterol [16]. The LM extract from SCFE-CO_2 at 36.8 °C, 100 bar with EtOH as a co-solvent at 0.99 mL/min indicated total phenolic content up to 0.82 mg GAE/mg dried LM [23]. Figure 4(c) depicts the initial and enlarged PVA/CS/LM extract/TEOS hydrogel film composites with time. The composite hydrogel film could maintain its shape for 60 min. However, after 30 min, it started to crack when it was contacted with the dissecting needle to transfer the film.

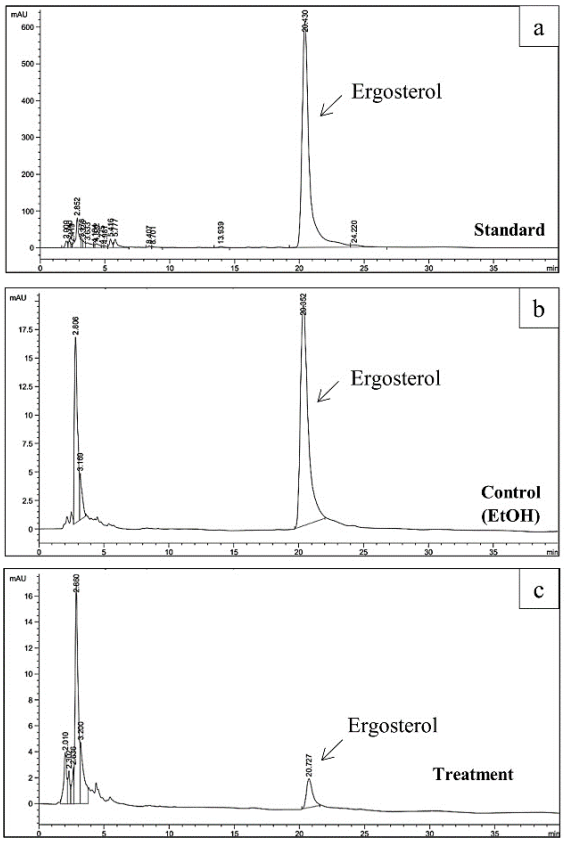

Figure 5: HPLC chromatogram of ergosterol standard (a), bioactive compounds released from PVA/CS/LM extract/TEOS hydrogel composites dissolved in ethanol (b), and phosphate buffer (c) after 300 min.

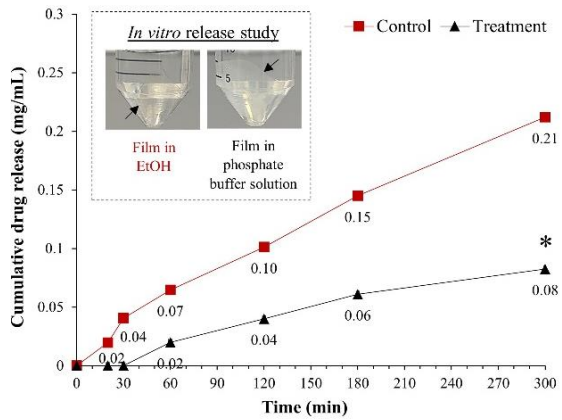

Figure 6: Cumulative release of ergosterol from PVA/CS/TEOS hydrogel film composites loaded with Lion's Mane mushroom extract submerged in ethanol (control), and phosphate buffer (treatment) at different times.

A PVA/CS/TEOS hydrogel film composites containing LM extract could increase swelling capacity and consistency, a crucial characteristic demonstrating the potential for liquid absorption and drug delivery systems.

3.2.2 Releasing of ergosterol from composite hydrogel film

The composite hydrogel film with LM loading was immersed in a phosphate buffer solution to simulate the human skin. EtOH was used to dissolve ergosterol from the film samples to determine the LM extract content in the film. Figure 5(b) and (c) show the HPLC chromatogram of ergosterol released from PVA/CS/LM extract/TEOS hydrogel film composites (Figure 5(b)) after soaking in EtOH and phosphate buffer (Figure 5(c)) for 300 min, compared to the ergosterol standard (Figure 5(a)). Cumulative releases of ergosterol from composite hydrogel film with the loading of LM extract with time are presented in Figure 6. The releasing rate of ergosterol in the buffer was higher during 30 min to 60 min after the submersion and declined afterward. The cumulative amount of ergosterol released increased with immersion time.

The results showed that ergosterol release from PVA/CS/LM extract/TEOS hydrogel film composites into a phosphate buffer solution, at 0.08 mg/mL, was about 38.93% compared to the ergosterol content in the composite hydrogel film (0.21 mg/mL) at 300 min of the immersion. Slow drug release from composite hydrogel film can improve drug efficacy, reduce the large drug dosage in the human body, and provide an optimal therapeutic effect [49]. Similar results were reported by Thanyacharoen *et al.*, [13] that PVA and CS composite films loaded with black rice extract exhibited a burst release at 60 min after submersion.

A PVA/CS/TEOS hydrogel film composites containing LM extract in this study had the ability to release ergosterol in phosphate buffer, which was increased with the immersion time (starting at 30 min). However, the release of ergosterol from the film was declined after 60 min. This was related to the swelling ability of PVA/CS/LM extract/TEOS hydrogel film composites. After 30 min, PVA/CS/LM extract/TEOS hydrogel film composites started to crack when it was contacted with the dissecting needle to transfer the film, which caused the film to have more surface area to attract to water molecules and tend to decline the solubility and releasing of ergosterol.

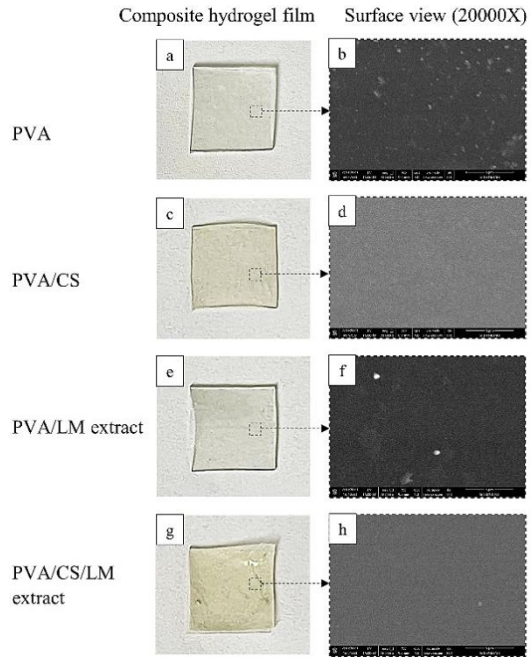

Figure 7: Morphology of polyvinyl alcohol (PVA) and chitosan (CS) hydrogel film composites loaded with Lion's Mane mushroom (LM) extract without crosslinker tetraethoxysilane (TEOS).

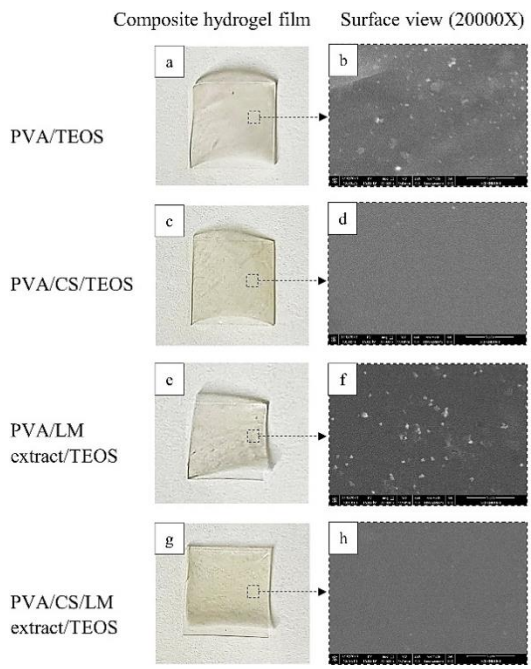

Figure 8: Morphology of polyvinyl alcohol (PVA) and chitosan (CS) hydrogel film composites loaded with Lion's Mane mushroom (LM) extract with crosslinker tetraethoxysilane (TEOS).

3.2.3 The color and appearance of composite hydrogel film

The color and appearance of each casted composite hydrogel film with/without TEOS before and after drying are shown in Figures 7 and 8. The composite hydrogel film prepared from PVA and CS (Figures 7(d) and 8(d)) showed a more homogeneous and uniform structure without significant holes compared to the film prepared from PVA only (Figures 7(b) and 8(b)), even though the TEOS was used or not. This demonstrated the excellent miscibility of PVA and CS. However, using TEOS led to the PVA/CS hydrogel film composites having a smoother and more flexible surface than the film without TEOS.

The formation of polyanion-cation complexes of PVA, CS, and TEOS in the hydrogel substantially impacted the crosslinking network [13]. Loading with LM extract to PVA/CS hydrogel film composites (with/without TEOS) displayed a homogenous, uniform structure without noticeable holes or roughness, as shown in Figures 7(h) and 8(h). This proved that the bioactive component in the LM extract was dissolved with PVA and CS.

The optical color of consumer products is a significant criterion for purchasing determination since it affects consumer desirability and acceptability [50]. When LM extract was added to PVA and PVA/CS hydrogel film composites without TEOS (Figure 9(a)), the film lightness (L^*) considerably decreased (darker) compared to the composite hydrogel films without extract loading. It can be described that some of the light wavelengths were absorbed by the pigments in the LM extract, leading to a lower content of the diffracted light and L^*. However, when TEOS was used in the film preparation (Figure 9(a)), adding LM extract led to a higher L^* (higher whiteness) than the film with no extract loading. The addition of TEOS greatly influenced the color of PVA/LM extract and PVA/CS/LM extract-hydrogel film composites. Cho *et al.*, [51] studied the effect of surface modification of ZrO_2 nanoparticles and using TEOS to produce transparent nanocomposite films with adjustable refractive index. They found that the light absorption by the SiO_2 layer of nanocomposite film was negligible. The nanocomposite film obtained demonstrated excellent optical transparency ($T_{average}$ = 91.1%), nearly comparable to the coverslip's transparency ($T_{average}$ = 91.4%). The color values, a^*, b^*, and L^*, of the films from each treatment are presented in Figure 9. The color values can evaluate the magnitude of the chromatic aberration and its

visual difference. However, the film color of almost all treatments did not show a noticeable color difference (ΔE) compared to the PVA hydrogel film composites (control).

3.2.4 Characterization of hydrogel film composites by FTIR spectroscopy

The FTIR spectra of LM extract-loaded PVA/CS hydrogel films composites are presented in Figure 10. The broad peak at 3300 cm^{-1} and 2925 cm^{-1} representing the hydroxyl group (O-H stretching) and the methyl group (C-H stretching) of inter and intra-molecular hydrogen bonding of PVA and CS were observed in all samples (Figure 10(b)). The spectra of PVA/CS/TEOS and PVA/CS/LM extract/TEOS hydrogel film composites showed a difference in the intensity of the peak as compared to other treatments. The 1100–1020 cm^{-1} peaks indicate siloxane linkage (Si-O-Si linkages), the bonding that can occur with PVA and CS. The peak of siloxane linkage was seen only in PVA/CS/TEOS and PVA/CS/LM extract/TEOS hydrogel composites. However, the treatment with TEOS did not exhibit distinguishable FTIR spectra in the Si-O-Si peak; this might be because of an incomplete crosslinking, as evidenced by the low swelling and low gel-forming stability of the films (Figure 4). The stability of hydrogel films depended on the equilibrium between the assembled network and the dissolved gelators. More research should be done to determine the optimal PVA, CS, LM extract, and TEOS ratio.

This phenomenon confirmed the crosslinking reaction between the PVA/CS hydrogel composites containing TEOS. The intensity of the siloxane linkage peak was the widest and highest in the PVA/CS/TEOS hydrogel film composites. It decreased when adding the LM extract, which means other bonding occurred between the LM extract and the polymer inside the composite hydrogel films. A PVA/CS/LM extract/TEOS hydrogel film composites had the characteristics that might be suitable for wound-healing applications due to its compact and homogeneous properties, maintaining its shape, liquid absorption, and the release of ergosterol from the film.

In recent years, there have been some studies on semiconductor ingredients, including CuZn, for use as biosensors in targeted therapy. The described drug delivery systems demonstrated precise delivery of antitumor efficacy to trigger releases of active pharmaceutical ingredients [52]–[54]. Alginate copper oxide was used as a glucose biosensor in the biocomposite film, while cellulose nanofiber was applied in aerogel as a bacterial biosensor [55]. In addition, tissue engineering is interesting as an alternative structural strength for scaffold supports in the biomedical area [56]–[58]. This PVA/CS/LM extract/TEOS hydrogel film composites in this study indicated potential development into hydrogel film with biosensors for drug delivery and biomaterials for tissue-implanted scaffold applications.

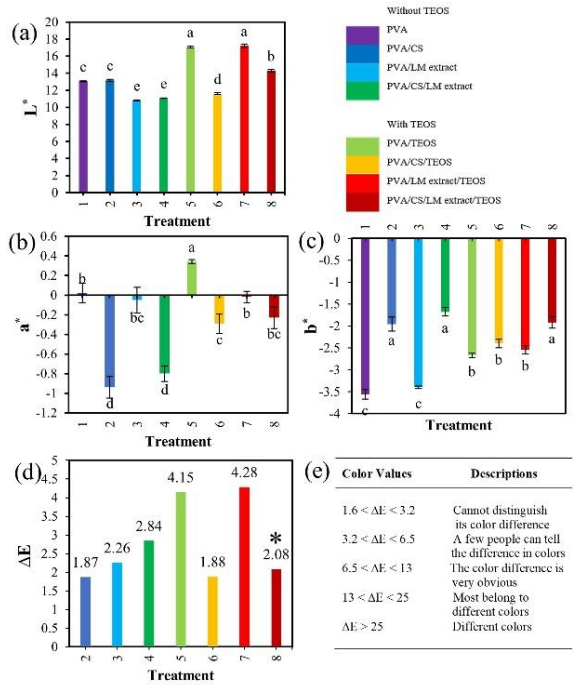

Figure 9: Color measuring of polyvinyl alcohol (PVA) and chitosan (CS) hydrogel film composites loaded with Lion's Mane mushroom (LM) extract with/without crosslinker tetraethoxysilane (TEOS): (a) L*, lightness/brightness; (b) a*, redness/greenness; (c) b*, yellowness/blueness; (d) ΔE, total color difference; (e) General color values and their corresponding descriptions. Data are expressed as mean values of 4 replications ± SD (standard deviation). Different superscripts in the same column of each bar mean significant difference at p-value < 0.05.

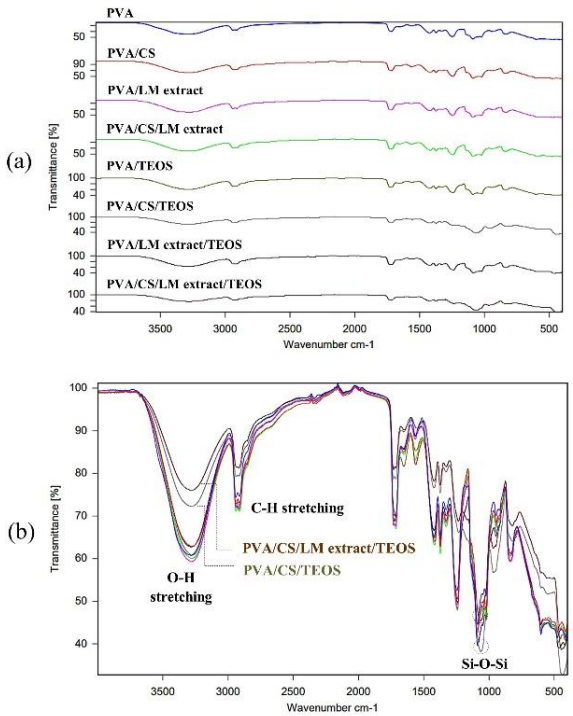

Figure 10: Stack curve (a) and overlay curve (b) of FTIR spectra from polyvinyl alcohol (PVA) and chitosan (CS) hydrogel film composites loaded with Lion's Mane mushroom (LM) extract with/without crosslinker tetraethoxysilane (TEOS).

4 Conclusions

This work established the therapeutic effects of SCFE-CO_2 LM extract on *in vitro* wound-healing. At a 1 mg/mL extract dosage was not toxic to fibroblast cells, cell viability ranged from 94.60–97.40%, and could promote cell proliferation at the site of wound scratching. The HDF cell migration at the scratch line with the LM extract after 48 h of the treatment was faster than the control (10% v/v DMSO and DMEM). The structural properties of the composite hydrogel films were evaluated using FTIR and SEM, which showed good miscibility between PVA, CS, and the extract and presented a compact and homogeneous film. PVA/CS/LM extract/TEOS hydrogel film composites could maintain their shape for 60 min with 625% of the swelling ratio. The rate at which ergosterol was released from the film was comparatively greater after the immersion in phosphate buffer for 30 to 60 min. Comparing the film colors of all treatments to the PVA hydrogel film composite (control), no noticeable color variation was seen (ΔE). TEOS was used as a crosslinking agent to improve the swelling ability. Remarkable results confirmed the use of composite hydrogel films prepared from PVA, CS, and TEOS for the loading of bioactive compounds from LM extract and applied for wound-healing hydrogel film composites in medicinal applications. LM used in this study were the by-products obtained after trimming the LM for packing. Hence, preparing the LM extract and encapsulation into the biofilm for medical purposes would value adding the cheap by-products. In addition, further studies on the development of LM extract into functional foods would also benefit future food trends. The traditional uses of lint, cotton bandages, or gauze without active ingredient loading usually take a long time for the wound to heal. Therefore, more studies are required to explore novel modalities with high treatment efficiency. It is not easy to maintain the stability and quality of hydrogel films. The dose of bioactive chemicals may be the limiting factor, which must be further investigated. More research should be done on the enhancement of swelling capacity, extending the longer release rate of LM extracts, and the *in vitro* and *in vivo* study on the use of the hydrogel films.

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Author Contributions

P.J.: conceptualization, investigation, reviewing, and editing; V.R.: investigation, methodology, writing an original draft; U.R.: research design, data analysis; B.T.: conceptualization; S.V.: conceptualization; K. S.: conceptualization; A.C.M.: investigation, methodology, writing an original draft.

Conflicts of Interest

The authors declare no conflict of interest.

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