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PREPARATION AND CHARACTERIZATION OF GELATIN-BASED HYDROGELS ENCAPSULATING CENTELLA ASIATICA EXTRACT FOR BIOMEDICAL APPLICATIONS

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Abstract. Exploiting an inherently biological activity of natural compounds in *Centella asiatica*, a new hydrogel system with many beneficial properties was developed by a simple preparation. Gelatin-based hydrogels were *in situ* formed by Schiffbase reaction to incorporate a *Centella asiatica* extract. The gelation time was controlled from 1 to more than 30 minutes by varying a composition ratio. The gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogels exhibited microarchitecture and high porosity when being observed with a scanning electron microscope. The swelling ratio was reported in the range from 106 % to 121 %, which was an acceptable value for biomedical usage without any adverse effect on system blockage or stress. In addition, all hydrogels formed with less than or equal to 0.5 wt% glutaraldehyde showed high cell compatibility; a *Centella asiatica* extract (≤ 1 wt%) did not cause any effects on cell toxicity. Interestingly, the hydrogels composed of 2.5 wt% gelatin, 0.25 wt% extract, and 0.25 wt% glutaraldehyde gained 105 % cell viability compared to standard culture conditions. Altogether, the gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogels could become a promising candidate for tissue engineering and drug delivery platform.

Keywords: hydrogels, *Centella asiatica*, drug delivery, cytotoxicity.

Classification numbers: 2.7.1.

1. INTRODUCTION

Hydrogels have gained special attention in various biomedical applications because of their unique properties and many advantages. Hydrogels are three-dimensional polymeric networks and able to contain a large water amount [1]. It is easy to recognize that their structure is very similar to the native extracellular matrix. Hydrogels have high biocompatibility, possess a biodegradable ability, can carry and sustainably release various categories of therapeutic agents. Moreover, hydrogel characteristics can be easily controlled through the choices of precursor polymers, crosslinkers, and many other additives. Also, hydrogels can be fabricated as injectable

materials [2], which provide the ability to fill the irregular space of wounds, adhere to tissues, and *in situ* encapsulate cells/drugs [1].

In recent years, many studies related to appropriate engineering hydrogels for biomedical applications such as wound dressings, drug delivery scaffolds, and tissue regeneration have been carried out [1, 3-5]. Gelatin-based hydrogels are widely used because gelatin possesses good bioactivity, non-toxicity, ease of modification, and require mild processing conditions [3, 4]. Gelatin-hydrogels have been formed by a variety of chemical reactions using glutaraldehyde, 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS), and diisocyanate [3]. This method was the simplest. Gelatin was exploited its inherent amine groups to do reactions without any modification. Besides, gelatin could be functionalized with methacrylate groups, then polymerized by ultraviolet light and initiators [3]. Another study reported the gelatin functionalized with phenol moieties, and then a horseradish peroxidase/hydrogen peroxide-catalyzed reaction was applied to form hydrogels [6]. Although chemical crosslinker such as glutaraldehyde is toxic, it can be controlled to reduce its amount within an acceptable range. Then this method may be more straightforward than others, and the complicated synthesis was minimized to save time, cost and to protect the environment from pollution.

Centella asiatica belongs to the *Umbellifere* family, known as Gotu kola [7]. It is one of the herbal plants understood and applied in traditional medicine for a long history. Many types of metabolites have been identified for both their chemical structures and therapeutic activities [7, 8]. Triterpene pentacyclic saponins are secondary metabolites having double structural characteristics, including a hydrophobic cyclic triterpenoid structure and sugar chains. This phytochemical family is known as a protective substance because of its antifungal and antimicrobial ability against pathogen infections [7]. Besides, *Centella asiatica* contains other triterpenoid saponins, such as asiaticoside, madecassoside, brahminoside, centelloside, centellose, brahmoside, sceffoleoside, madecassoside acid, and various other acids [8]. Asiaticoside is a component with known pharmacological activities, including wound healing ability, vasodilation, antiinflammatory action, antioxidant improvement, prevention of platelet aggregation, and vascular endothelium protection [8]. Madecassoside acid exhibited its neuroprotective ability in rat models [9]. In addition, *Centella asiatica* contains tannins, an essential acid, phytosterols, mucilages, free amino acids, mucilages, flavonoids, and several bitter components [8, 9]. Altogether, *Centella asiatica* with abundant natural substances covers a wide range of biological activities, including wound healing, neuroprotective, antitumor, antibacterial, antifungal, antioxidant, antiulcer, and antiinflammatory ability [8, 9].

To apply those excellent bioactivities of *Centella asiatica*, the scaffold carrying its natural substances is required to protect them from degradation and create a sustainable release to improve efficacy. Lee *et al.* used the preformed hydrogels composed of β-cyclodextrin-grafted polyethyleneimine, silk fibroin, and crosslinker of epichlorohydrin (β-CD/PEI/SF)[10] to co-deliver a *Centella asiatica* extract and hydrocortisone acetate. This formulation was tested *in vivo* and showed enhanced wound healing. However, the preformed hydrogels would be limited in some applications compared to the injectable forms. The drugs/compounds loaded in β-CD/PEI/SF hydrogels by the immersing method achieved limited amounts due to equilibrium osmosis. Ahmed *et al*. prepared the polyvinyl alcohol/polyethylene glycol (PVA/PEG) hydrogels loading *Centella asiatica* extract using the freeze-thaw method. These *Centella asiatica*/PVA/PEG hydrogels induced a faster epithelisation period when tested on rabbit wound healing model. However, the usage of PEG could cause the generation of anti-PEG antibodies, leading to a decrease in the efficacy and an increase in the side effects of PEGylated therapeutics

[11, 12]. Recently, Hong *et al*. developed a *Centella asiatica* extract-loaded hyaluronic acid-dextran hydrogels (CAE-HA-Dex) using a freeze-drying proccess for atopic dermatitis treatments [13]. The CAE-HA-Dex hydrogels could effectively provide moisture for wounds, release the bioactive compounds for cellular activity, and relieve atopic itching. Their limitations were the long fabrication process and preformed types. Therefore, the designation of a suitable scaffold for encapsulation of *Centella asiatica* extract should be developed to overcome the current limitations and obtain further achievement.

Grasping those benefits of *Centella asiatica* and hydrogels, we would offer a new hydrogel system. Aqueous *Centella asiatica* leaf extract (aCAL) was prepared and qualitatively tested for its phytoconstituents. Gelatin-based hydrogels were fabricated by Schiffbase reaction to load a CAL containing bioactive agents. The multicomponent hydrogels are expected to achieve synergistic and complementary effects, resulting in a valuable contribution for biomedical applications. The gelatin/aCAL-glutaraldehyde hydrogels were prepared, characterized, and tested for toxicity. The gelation time was determined by the vial tilting method. Ninhydrin assay was used to indirectly explore the reaction between primary amine groups of gelatin and aldehyde to form imine linkages. The porosity of hydrogels was observed by scanning electron microscopy. The swelling ratio of hydrogels was investigated. Human dermal fibroblast viability percentage after contacting with various hydrogel extracts was accessed by WST-1 assay.

2. MATERIALS AND METHODS

2.1. Materials

Gelatin from porcine skin (gell strength 300, type A), magnesium foil (99.9 %), ninhydrin reagent, potassium chloride (KBr), and glutaraldehyde solution (grade I, 25 % in H_2O) were bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Acetic anhydride (ACS, Reag. Ph. Eur) was purchased from Prolabo (VWR Chemicals, France).

Iodine (Analytical reagent (AR)), potassium iodide (AR), copper (II) sulfate pentahydrate (AR), concentrated sulfuric acid, concentrated hydrochloric acid, ferric chloride (AR), sodium hydroxide (AR), and potassium sodium tartrate (AR), and chloroform were obtained from Xilong Scientific Co., Ltd. (Shantou, Guangdong, China).

Dulbecco's Modified Eagle's Medium - high glucose (DMEM), penicillin-streptomycin (P/S), and trypsin/EDTA were bought from Gibco BRL (Grand Island, NY, USA). Dulbecco's phosphate-buffered saline (DPBS) and fetal bovine serum (FBS) were obtained from Wisent (Saint-Bruno, QC, Canada). WST-1 assay reagent (EZ-Cytox enhanced cell viability assay kit) was supplied by ITSBIO (Seoul, South Korea).

Leaves of *Centella asiatica* were collected from Binh Chanh district, Ho Chi Minh city, Vietnam in the period from July to September 2020. All leaves which must be matured and healthy were washed carefully to discard dusts and other contaminants. *Centella asiatica* leaves were dried under sunlight until their humidity was less than 5 weight percentage (wt%). This material plant was cut into small pieces. Maceration was performed in a conical flask containing cut leaves (150 g) and water (300 mL) at 80 - 90 ^{o}C for 8 hours. This procedure was repeated three times to accumulate high amount of extract. All collected extracts were lyophiolized to obtain dried powder symbolized as aCAL.

2.2. Phytochemical screening of aqueous *Centella asiatica* leaf extract (aCAL)

Shinoda test was utilized to recognize flavonoids [14]. A few fragments of magnesium metal were added to the crude extract aCAL solution. Concentrated hydrochloride acid was dropped into this mixture. After a few minutes, if the pink scarlet color occurred, flavonoids were present in aCAL.

Wagner test was performed to identify the presence of alkaloids [14]. Wagner's reagent was prepared from an iodine and potassium iodide solution. If aCAL reacts with Wagner's reagent forming a brownish precipitate, alkaloids were present.

Foam test was carried to identify saponins [15]. An aCAL solution was added to a test tube. After shaking well, the appearance of froth on the solution surface stood for at least 5 minutes, indicating the presence of saponins.

Liebermann-Burchard test was performed to identify steroids [14]. An aCAL solution was added to a test tube. Acetic anhydride was dropped into the aCAL solution. The mixture in the test tube was boiled and cooled to room temperature. Concentrated sulfuric acid was added from the test tube side. If the upper layer showed green color, steroids were present.

Ferric chloride solution was utilized to detect the presence of polyphenols [16]. If the aCAL solution reacted with ferric chloride creating a blue, green, violet, or red-brown color, polyphenols were present in the aCAL crude extract.

Biuret test was performed to identify proteins [17]. In a test tube, the aCAL solution reacted with copper (II) sulfate in a sodium hydroxide and potassium sodium tartrate solution. If there was the formation of mauve-colored complexes, proteins were present.

Salkowski test was used for triterpenoid identification [18]. In a test tube, an aCAL solution and chloroform was mixed in a volume ratio of 1:1. A few drops of concentrated sulfuric acid were dropped into the test tube. After shaking homogenously, if there was the formation of a yellow layer, triterpenoids were present.

2.3. Hydrogel formation and characterization

Table 1. The hydrogels formulated from gelatin (Gel), aqueous extract solutions of *Centella asiatica* leaves (aCAL), and glutaraldehyde (GDA).

Hydrogel code	Final gelatin concentration (wt%)	Final glutaraldehyde concentration (wt%)	Final aCAL concentration (wt%)
Gel5/aCAL1-GDA1	5	1	1
Gel5/aCAL1-GDA0.5	5	0.5	1
Gel5/aCAL1-GDA0.25	5	0.25	1
Gel5/aCAL0.5-GDA1	5	1	0.5
Gel2.5/aCAL1-GDA0.25	2.5	0.25	1
Gel2.5/aCAL0.5-GDA0.25	2.5	0.25	0.5
Gel2.5/aCAL0.25-GDA0.25	2.5	0.25	0.25
Gel2.5/aCAL0.5-GDA1	2.5	1	0.5

The hydrogels were fabricated from gelatin (Gel), aCAL solution and glutaraldehyde (GDA) crosslinkers. Gelatin solutions were prepared in warm distilled water (DIW) at initial

concentrations of 5 and 10 wt%. Glutaraldehyde of 25 wt% was diluted to various initial concentrations, including 1.25, 2.5, and 5 wt%. Aqueous extract solutions of *Centella asiatica* leaves (aCAL) were prepared in DIW at different initial concentrations (1.25, 2.5, and 5 wt%). To form the hydrogels, 250 μL of 10 wt% gelatin, 100 μL of 5 wt% aCAL, 100 μL of 5 wt% GDA, and 50 μL of DIW were added to an Eppendorf tube and mixed homogeneously to fabricate 500 μL of Gel5/aCAL1-GDA1 hydrogels. The numerical part in hydrogel codes indicated the final concentration of each component in the obtained hydrogels. Similarly, other Gel/aCAL-GDA hydrogels with different compositions, as shown in Table 1, were fabricated.

The gelation time of hydrogels was measured by a vial tilting method [2, 19]. After adding a GDA solution to the Gel/aCAL mixtures, the mixtures were observed to record their gelling points where the liquids could not flow. Each sample was triplicated to calculate the average value and standard deviation.

A ninhydrin test was performed to identify the amine groups after hydrogel formation [2]. A ninhydrin solution of 2 wt% was prepared in ethanol. In the test tubes, 500 µL of hydrogels was formed with the components listed in Table 1. After 60 minutes, all hydrogels were cut into small pieces. 1 mL of the ninhydrin solution was added to all cut hydrogel samples. These ninhydrin reactions were carried out in a water bath at 80 ^{o}C for at least 20 minutes. The purple color indicated the presence of amine groups, the color density was proportional to the amine amount.

The lyophilized Gel5/aCAL1-GDA1 hydrogels were ground with KBr at a weight ratio of 1:100. The mixture was pressed into a transparent pellet. Fourier-transform infrared spectroscopy (FTIR, PerkinElmer Spectrum 10.5.2, Waltham, MA, United States) was operated at a wavelength number from 4000 to 400 cm^{-1} to obtain their FTIR spectrum.

For swelling ratio measurement, 500 µL of each hydrogel in Table 1 was formed in an Eppendorf tube. The lyophilized hydrogel samples were immersed in 10 mL of phosphate buffer saline (PBS, pH = 7.4) at 37 ^{o}C [20]. After 24 hours, the hydrogel samples absorbed enough water to reach the equilibrium state. The weights of lyophilized hydrogel (W_o) and of water-absorbed hydrogel at the 24th hour (W_w) were recorded to calculate the swelling ratio of hydrogel according to Equation (1). The calculation of each swelling ratio value of hydrogel was done in triplicate to obtain the average value and standard deviation.

$$Swelling\ ratio\ (\%) = \frac{W_w}{W_o} \times 100\% \quad (1)$$

A scanning electron microscope (FE-SEM, S-4800, Hitachi, Okinawa, Japan) was used to observe the porosity of the lyophilized hydrogels at a low voltage of 1 kV.

2.4. Cytotoxicity test

Human dermal fibroblasts (1 mL, 5 × 10^4 cells/mL) were seeded on a 24-well plate in DMEM-PS-10 % FBS media, at 37 ^{o}C, 5 % CO_2 and 95 % huminidity for 3 days. Four hydrogels, including Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, Gel2.5/aCAL0.25-GDA0.25, and Gel2.5/aCAL0.5-GDA1, were fabricated in an Eppendorf tube, the total volume of each sample was 250 µL. Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, and Gel2.5/aCAL0.25-GDA0.25 hydrogels with the same gelatin and glutaraldehyde contents were used to investigate the effect of the aCAL extracts. Gel2.5/aCAL0.5-GDA1 hydrogels with higher glutaraldehyde concentration than Gel2.5/aCAL0.5-GDA0.25 ones was tested to understand the cytotoxicity of glutaraldehyde. After 60 minutes of stabilization, DMEM was added to each hydrogel sample and incubated for 24 hours. These hydrogel extracts were used to

treat the hDFBs growing on the 24-well plate. The hDFBs in DMEM-PS-10 % FBS media which were not treated with hydrogel extract were a control sample. The proliferation of hDFBs after 24-hour treatment and without treatment with hydrogel extracts was accessed by WST-1 assay. All media were removed, and then washed carefully with DPBS three times. Fresh DMEM (1 mL) and 100 µL of WST-1 reagent were added into hDFB wells. A blank was prepared from fresh DMEM (1 mL) and 100 µL of WST-1 reagent without hDFBs. After 2 hours of incubation, the absorbence of each reacted mixture was measured by a microplate reader (Cytation™ 3 Cell Imaging Multi-Mode Reader, BioTek™, VT, USA) at 450 nm. The percentage of hDFBs viability was calculated according to formula (2).

$$\%\ viability = \frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}} \times 100\%. \quad (2)$$

2.5. Statistical analysis

Results were calculated and expressed as the mean ± standard deviation. Statistical analyses were performed by the Student's t test. Statistical differences were considered to be significant when P-value was less than 0.05.

3. RESULTS AND DISCUSSION

3.1. Phytoconstituents in an aqueous *Centella asiatica* leaf extract (aCAL)

The Shinoda, Wagner, foam test, Liebermann-Burchard, ferric chloride test, Biuret test, and Salkowski test were applied to identify the flavonoids, alkaloids, saponins, polyphenols, proteins, and triterpenoids in aCAL, respectively. After testing, the Shinoda test, foam test, ferric chloride, and Salkowski test showed the positive phenomenons in the tested aCAL solutions, including the appearance of pink color, stable froth, dark blue color, and yellow layer, respectively. These results indicated that the aCAL contained flavonoids, saponins, polyphenols, and triterpenes; vice versa, alkaloids, steroids, and proteins were not found in the aCAL (Table 2). So, aCAL was proven to have many kinds of excellently bioactive compounds playing a potential role in variously biomedical applications. These results were consistent with the studies of Sekar *et al.* [21], except for the presence of saponins. However, the confirmation of saponins in our study corresponded with many other reports. Sampson *et al.* demonstrated that the triterpenoid saponins in an aqueous *Centella asiatica* leaf extract could show an *in vitro* keratinocyte antiproliferant effect [22]. Irham *et al.* and Wu *et al*. reported triterpene saponins in aCAL exhibiting wound healing [23] and neuroprotective effects [24]. One of the triterpenoid saponins found in *Centella Asiatica* was asiaticosides structured by the aglycone asiatic acid, madasiatic acid, and madecassoside linked to a trisaccharide moiety [25]. In addition to its bioactivities mentioned above, this primary active phytoconstituent exhibited vascular and ulcer-reducing effects [25]. Flavonoids are also meaningful substances in the circulatory system, blood vessels, and anti-inflammatory properties [8]. In addition, polyphenols can show their high antioxidant potential for antiaging effects, treatment of reactive nitrogen species, and reactive oxygen species-generating diseases. For more details, the specific compounds of each family were also identified. Triterpenes and saponins can be asiaticoside and its derivatives, braminoside, brahmoside, brahminoside, thankuniside, isothankuniside, entellasaponin and its derivatives, centellasapogenol, centelloside, and madecassoside [9]. Flavonoids and polyphenols include quercetin glycoside, astragalin, rutin, kaempferol, catechin, and naringin [9]. Thus,

aCAL is a potential multicomponent for encapsulation within gelatin hydrogel matrices applied for wound healing and other biomedical fields.

Table 2. Phytochemical screening of aqueous *Centella asiatica* leaf extract (aCAL).

Phytoconstituent	Method	Phenomenon	Results
Flavonoids	Shinoda test	Pink color	+
Alkaloids	Wagner Test	None	-
Saponins	Foam test	Stable froth	+
Steroids	Liebermann-Burchard	None	-
Polyphenols	Ferric Chloride test	Dark blue color	+
Proteins	Biuret test	None	-
Triterpenoids	Salkowski test	Yellow layer	+

3.2. Hydrogel formation and characterization

Gelatin solutions were mixed with aCAL first and vortexed to distribute the bioactive compounds homogeneously. Then glutaraldehyde (GDA) was added to crosslink gelatin backbones together through Schiffbase reaction. Gelatin possessing primary amine groups could interact with aldehyde groups to form imine linkages (Figure 1a), resulting in the formation of gelatin/aCAL-GDA hydrogels encapsulating the bioactive compounds mentioned above inside the gel matrices.

The formation of imine linkages was indirectly examined through the Ninhydrin test [2]. The primary amine groups interact with ninhydrin, forming purple products. Besides, ninhydrin also reacts with secondary amines to form yellow-orange iminium salts . Therefore, the observation of reacted solutions, including gelatin/aCAL-GDA hydrogels and ninhydrin reagents, could forecast amine residues. Figure 1b shows the results of ninhydrin tests for various hydrogels. Three hydrogels, including Gel5/aCAL1-GDA1, Gel5/aCAL0.5-GDA1, and Gel2.5/aCAL0.5-GDA1 reacted with ninhydrin and generated yellow solutions that implied that there were no primary amine residues and the reaction was completed. In the case of Gel5/aCAL1-GDA0.5, Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, and Gel2.5/aCAL0.25-GDA0.25, the light purple colour was observed. Among all hydrogels, the Gel5/aCAL1-GDA0.25 hydrogels exhibited the darkest purple. These results revealed that the amine groups of gelatin reacted with glutaraldehyde to form imine linkages; thus no or less primary amine was detected. The 1 wt% glutaraldehyde could completely react with 5 wt% gelatin, while lower glutaraldehyde amounts were insufficient to interact with entire amine groups of gelatin. In addition, the imine linkages within hydrogel matrices were confirmed by FTIR spectra (Figure 1c). The presence of a medium peak in the wavenumber range of 1630-1640 cm^{-1} was assigned to the C=N stretching vibration [26].

Gelation time is one of the important parameters for hydrogel applications. For each specific situation, different gelation times are required to prevent needle blockages or spread around adjacent tissues. Thus, the gelation time of various hydrogels was investigated to understand the exact value and governing factors. In Figure 2a, Gel5/aCAL1-GDA1, Gel5/aCAL1-GDA0.5, Gel5/aCAL1-GDA0.25 hydrogels could be gelled after 1.3, 1.7, and 3.0 minutes, respectively. These three formulations had the same gelatin and aCAL concentrations, their gelation time was slowed down with a decrease in glutaraldehyde concentration.

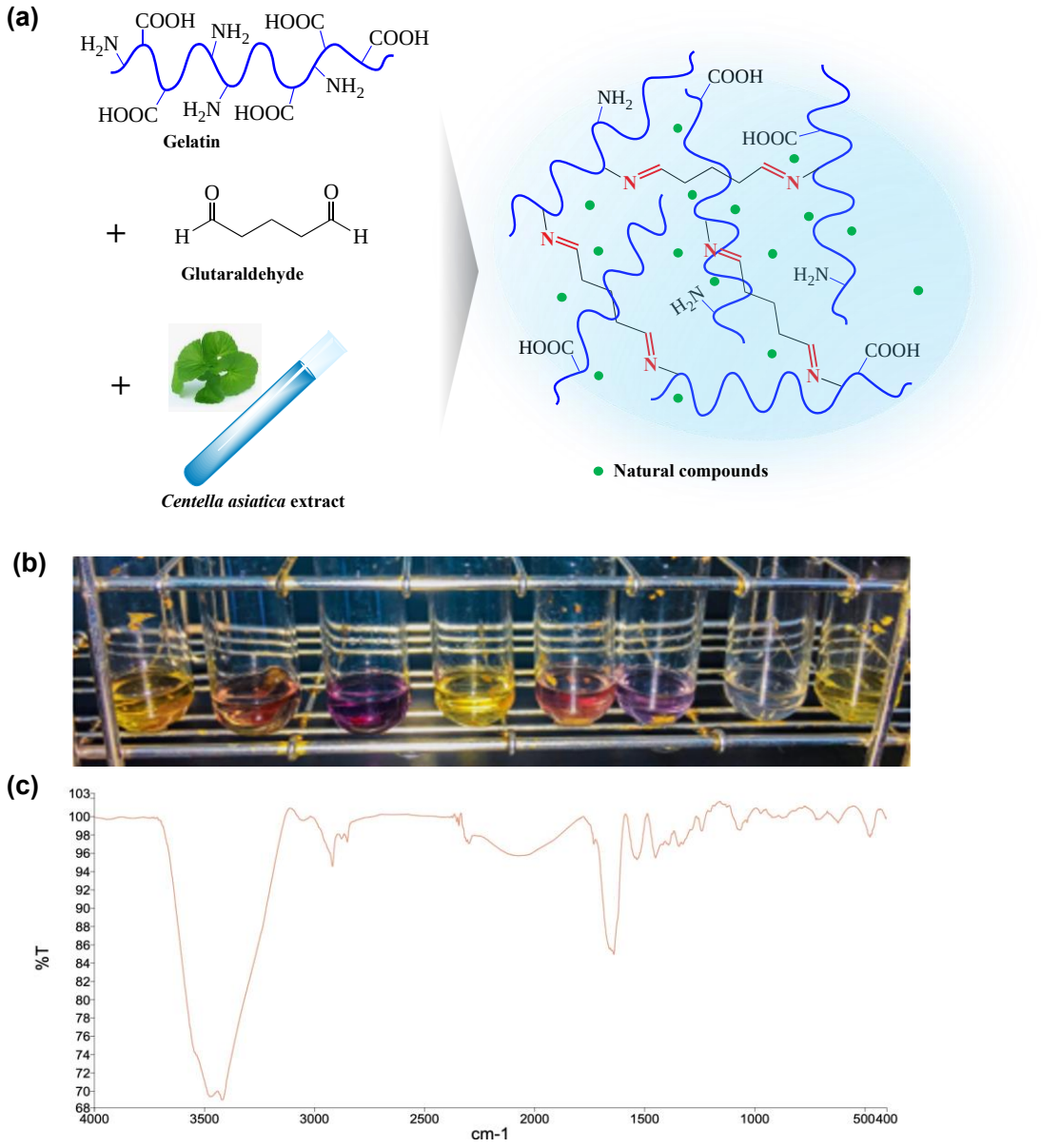

Figure 1. Schematic diagram of gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogel formation (a). Ninhydrin test of hydrogel samples, including Gel5/aCAL1-GDA1, Gel5/aCAL1-GDA0.5, Gel5/aCAL1-GDA0.25, Gel5/aCAL0.5-GDA1, Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, Gel2.5/aCAL0.25-GDA0.25, and Gel2.5/aCAL0.5-GDA1 (from left to right): the purple indicates the presence of primary amines (b). FTIR spectrum of Gel5/aCAL1-GDA1 hydrogels (c).

The gelation times of Gel5/aCAL1-GDA0.25 and Gel5/aCAL0.5-GDA1 hydrogels were similar, which indicated that different aCAL contents did not govern the gelation time if using 1 wt% GDA. It can be seen from Figure 2b that, Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, and Gel2.5/aCAL0.25-GDA0.25 hydrogels could be solidified after 30.2, 26.8, and 22.1 minutes, respectively. These hydrogels had the same gelatin and GDA amount, their

gelation time was more rapid if the aCAL amount was reduced. Taken together, aCAL content did not impact the gelation time only when using high amounts of glutaraldehyde. It might be due to the hindrance of small bioactive compounds to the reaction between gelatin and glutaraldehyde through hydrogen bondings or space effects. However, this reason becomes meaningless when the amount of GDA was large. The effect of aCAL on gelation time was less significant than that of GDA. Indeed, Gel2.5/aCAL0.5-GDA1 hydrogels achieved gelation 2.4 times faster than Gel2.5/aCAL0.5-GDA0.25 ones. When considering the gelatin amount between Gel5/aCAL0.5-GDA1 (Figure 2a) and Gel2.5/aCAL0.5-GDA1 (Figure 2b), it could be recognized that the higher gelatin could form hydrogels more rapidly due to more reaction centers and longer polymer chains. Overall, the gelation time of Gel/aCAL-GDA hydrogels was controlled from one to several minutes. Thus, it looks a little slower than the horseradish peroxidase-catalyzed hydrogels and the thiol-epoxy "click" ones formed within a few seconds to several minutes [27, 28]. However, it was more rapid than the gelation time of the oxidized methacrylate alginate/8-arm poly(ethylene glycol) hydrogels (5 – 25 minutes [29]) and microbial transglutaminase-catalyzed hydrogels (21 – 124 minutes [30]).

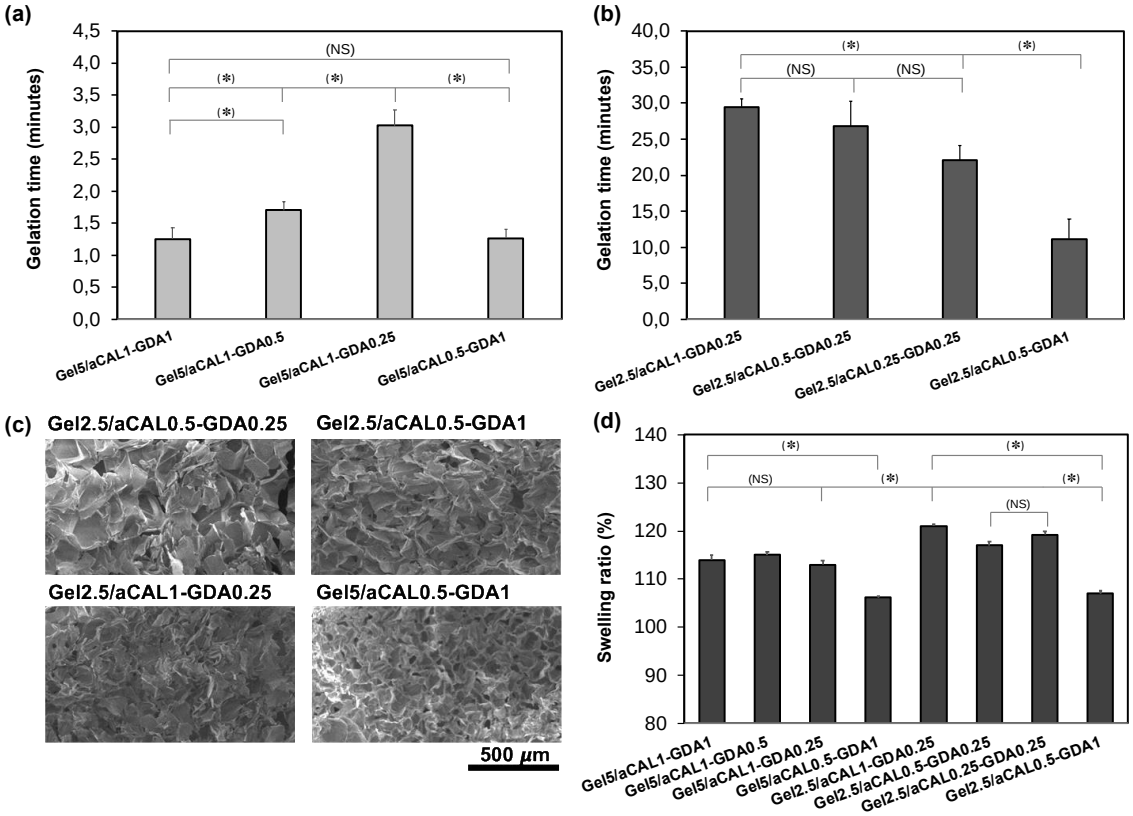

Figure 2. Gelation time of the five wt% gelatin (Gel)-based hydrogels with varied glutaraldehyde (GDA) and aqueous *Centella asiatica* extract (aCAL) amounts (a); Gelation time of the 2.5 wt% gelatin-based hydrogels with varied aCAL and GDA concentrations (b); The SEM micrographs of the cross-section of Gel2.5/aCAL0.5-GDA0.25, Gel2.5/aCAL0.5-GDA1, Gel2.5/aCAL1-GDA0.25, and Gel5/aCAL0.5-GDA1 hydrogels (c); The swelling ratio of lyophilized gelatin/aCAL-GDA hydrogels (d).

Figure 2c shows the cross-sectional images of various hydrogels observed by a scanning electron microscope. All hydrogel formulas, including Gel2.5/aCAL0.5-GDA0.25, Gel2.5/aCAL0.5-GDA1, Gel2.5/aCAL1-GDA0.25, and Gel5/aCAL0.5-GDA1 hydrogels, exhibited their microstructure and high porosity. Among them, the Gel5/aCAL0.5-GDA1 hydrogels showed the smallest pores because the highest concentrations gelatin and glutaraldehyde resulted in the formation of a tight cross-linking network. Gel2.5/aCAL1-GDA0.25 hydrogels had the largest pores due to the lowest crosslink density, this was because low glutaraldehyde was used and hydrogel formation was affected by high aCAL. The pore size of Gel2.5/aCAL0.5-GDA0.25 hydrogels was smaller than that of Gel2.5/aCAL0.5-GDA1 hydrogels due to a low concentration of glutaraldehyde forming a low crosslink density. Taken together, gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogels could be controlled for their porosity by varying gelatin, aCAL, and glutaraldehyde concentrations. The porosity of the scaffolds is an essential property in biomedical applications. This property supports the scaffolds to exchange nutrients, moist and oxygen homogeneously through the matrices. In addition, porosity is required for cell growth to create tissues.

The swelling ratio is another important characteristic of hydrogels and must be precisely known [31]. When hydrogels are implanted inside the human body, the swollen hydrogels can block the vascular system or put stress on nearby sites. If hydrogels are utilized as a bandage, the swelling parameter can reflect their absorption of exudate or pus. To understand the swelling state of gelatin/aCAL-GDA hydrogels, we measured the hydrogels' weight in the dried and swollen states, and their swelling ratio was shown in Figure 2d. Eight hydrogel formulas achieved their swelling ratio in the range of 106 – 121 %. Using the same glutaraldehyde (0.25 %) and aCAL (1 %) amounts, these Gel/aCAL-GDA hydrogels with 5 wt% gelatin gained a lower swelling ratio than that of hydrogels with 2.5 wt% gelatin (113 % vs 121 %). This can be explained that a higher gelatin concentration could form a higher crosslinking density, which hinders the absorption of water. Similarly, increasing GDA concentration led to an increase in crosslinking density and a decrease in swelling ratio. In the case of Gel2.5/aCAL0.5-GDA0.25 and Gel2.5/aCAL0.5-GDA1 hydrogels, when the concentration of glutaraldehyde increased from 0.25 to 1 %, the swelling ratio decreased from 117 to 107 %, respectively. However, Gel5/aCAL1-GDA1, Gel5/aCAL1-GDA0.5, and Gel5/aCAL1-GDA0.25 hydrogels showed an insignificant difference in swelling ratio inspite of decreasing glutaraldehyde concentration. This abnormal phenomenon could be explained by the high aCAL content which can govern the glutaraldehyde and gelatin reaction. Indeed, Gel5/aCAL1-GDA1 and Gel5/aCAL0.5-GDA1 hydrogels got swelling ratios of 114 % and 106 %, respectively. The decrease of aCAL content led to a decrease in the swelling ratio, inferring an increase in crosslinking density. This observation could be applied to the case of Gel2.5/aCAL1-GDA0.25 and Gel2.5/aCAL0.5-GDA0.25 hydrogels (with swelling ratio of 121 % vs 117 %). However, the decrease of aCAL from 0.5 to 0.25 % (Gel2.5/aCAL0.5-GDA0.25 vs Gel2.5/aCAL0.25-GDA0.25 hydrogels) caused an insignificant difference in the swelling ratio. It might be due to the small aCAL amount having little effect on crosslinking formation. Overall, these hydrogel systems swelled in an acceptable range (less than 121 %) which may be a candidate alternative to the commonly used polyethylene glycol-based hydrogels, with its significant swelling ratio being a notable drawback [32].

3.3. Cytotoxicity test of gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogels

The cytotoxicity of gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogels was tested with human dermal fibroblasts (hDFBs). The viability percentage of hDFBs after contacting with hydrogel extract was calculated and summarized in Figure 3a. There were three hydrogel extracts, including Gel5/aCAL1-GDA1, Gel5/aCAL0.5-GDA1, and Gel2.5/aCAL0.5-GDA1, which showed cell viability of less than 80 %, a value considered cytotoxic. The reason is that at high glutaraldehyde concentrations, the unreacted glutaraldehyde releases and causes cell death. Indeed, the cytotoxicity and immunological responses of glutaraldehyde were indicated in the literature [33]. So, minimizing the amount of glutaraldehyde was an important undertaking. On the contrary, gelatin is well-known as a biocompatible polymer. Gelatin contains important binding sites for cell attachment, possesses biodegradable properties, and does not cause toxicity in cells [34]. In addition, the aCAL solutions also were tested with hDFBs to understand their cytotoxicity level. Various aCAL concentrations (1, 2.5, 5, and 10 %) were used to treat the hDFBs. Figure 3b shows the cell viability percentage after incubation for 24 hours. The aCAL 1 and 2.5 % gained more than 80 % cell viability, being a compatible level. In the case of aCAL 5 and 10 %, cell viability decreased to 75 and 70 %, respectively. So, the aCAL concentration incorporated within hydrogel matrices should be less than or equal to 2.5 % so as not to slow down the cell proliferation. Herein, the contents of aCAL incorporated inside all hydrogels were less than or equal to 1 %. To explore the optimal glutaraldehyde for cell compatibility, Gel5/aCAL1-GDA0.5, Gel5/aCAL1-GDA0.25, Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, Gel2.5/aCAL0.25-GDA0.25 hydrogels were tested and they gained 87 %, 96 %, 93 %, 98 %, and 105 % of cell viability, respectively (Figure 3a). It was realized that low concentrations of glutaraldehyde of 0.25 – 0.5 wt% were enough for hydrogel formation and did not lead to the residues causing cell toxicity.

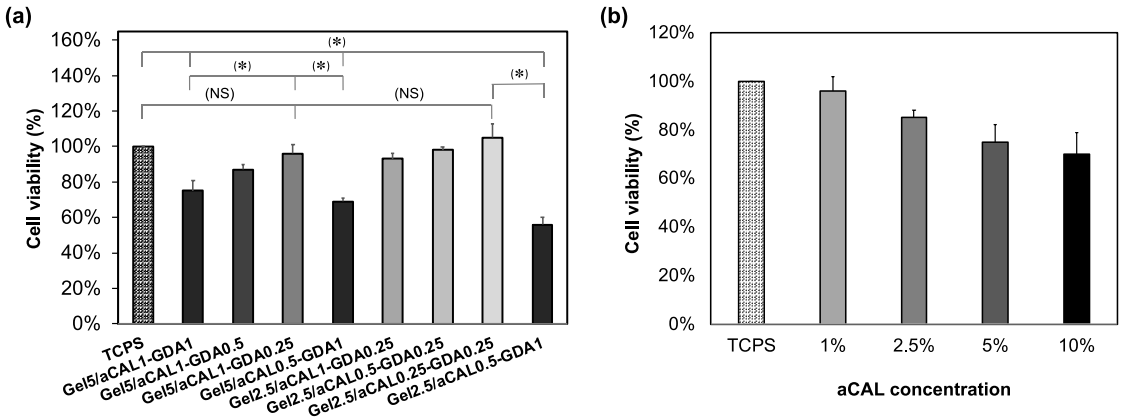

Figure 3. Viability percentage of human dermal fibroblasts (hDFBs) after incubating with various extracts of different gelatin/aqueous *Centella asiatica* leaf extract (aCAL)-glutaraldehyde hydrogels (a) and with different concentrations of aCAL (b) in comparison with hDFBs seeded on tissue culture polystyrene (TCPS) plate.

4. CONCLUSIONS

The gelatin hydrogels encapsulating aqueous *Centella asiatica* leaf extract were successfully synthesized with glutaraldehyde. Their gelation time was controlled from 1 to over 30 minutes by varying glutaraldehyde, gelatin, and *Centella asiatica* extract concentrations, but

the amount of extract had a significantly less governing effect than the other compositions. The results of scanning electron microscopy showed that the gelatin/aqueous *Centella asiatica* leaf extract-glutaraldehyde hydrogels possessed microarchitecture and high porosity. Their low swelling ratios were in the acceptable range from 106 % to 121 %. By investigating the cytotoxicity with human dermal fibroblasts, the optimal formulas of these hydrogels were found and determined to be free of cytotoxicity, including Gel5/aCAL1-GDA0.5, Gel5/aCAL1-GDA0.25, Gel2.5/aCAL1-GDA0.25, Gel2.5/aCAL0.5-GDA0.25, and Gel2.5/aCAL0.25-GDA0.25 hydrogels. Taken together, these hydrogel systems can become a promising green candidate for various biomedical applications in drug delivery, medicine regeneration, and tissue engineering.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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