CHAPTER V
PREPARATION, CHARACTERIZATION, AND ANTIBACTERIAL PROPERTIES OF GELATIN HYDROGELS PADS CONTAINING *EUPATORIUM ADENOPHORUM* ESSENTIAL OIL EMULSION

5.1 Abstract

Gelatin hydrogel was successfully prepared from a gelatin solution and essential oil, herbal substance from the plant *Eupatorium adenophorum* Spreng. which is commomly used in traditional medicine as antimicrobial activity. The oil in water emulsion *Eupatorium adenophorum* of essential oil were prepared and used Pluronic F68® as a surfactant. The 10-30 %v/v emulsion were mixed with gelatin solution and cast into film. Glutaraldehyde (GTA) was added to gelatin hydrogels incorporating with emulsion in order to crosslink this hydrogels. The emulsion-loaded gelatin hydrogels were investigated their gel fraction, swelling and weight loss behavior. With an increase in the emulsion concentration the emulsion-loaded in hydrogels, the gel fractions were decreased due to the crosslink density, while the swelling and weight loss behavior were increased with an increasing in the emulsion content. The emulsion-containing gelatin hydrogels were used as wound dressings and investigated the release behaviors of the loaded hydrogels, antimicrobial activity by using agar disk diffusion methods. The result showed that *E. adenophorum* essential oil and the emulsion-loaded inhibited the growth of the test pathogens. Lastly, *E. adenophorum* essential oil was identified the composition by using Gas chromatography-mass spectrometer (GC-MS) analysis and determined antimicrobial activities of essential oil in terms of minimum inhibition concentration (MIC) and agar well diffusion method. The main chemical composition of the oil was *p*-cymene (16.23%), bornyl acetate (11.84%), amorpha-4,7(11)-diene (10.51%). All of the emulsion-loaded gelatin hydrogel showed antibacterial activity with *S.agalactiae, A.calcoaceticus and S. epidermidis*. The inhibition zone of the as-loaded gelatin hydrogels were observed and increase in the higher amount of the emulsion in the hydrogels. These result indicated that the antibacterial activity of as-loaded gelatin hydrogels played important part in antimicrobial dressings.

(**Key-words**: Hydrogel; antibacterial wound dressing; *Eupatorium adenophorum* essential oil; gelatin)

5.2 Introduction

Hydrogels are polymer chains that have three dimensional networks and can be adsorb water of fluids. The ideal wound dressing material should protect the injury from infection, maintain the wetness and humidity in the wound area, and enhance the healing. (Peppas, Bures, Leobandung, and Ichikawa, 2000; Hoffman, 2002; Liu, et.al, 2005; Sokolsky-Papkovet.al. 2007; Hamidi, Azadi, and Rafiei, 2008; Singh and Pal, 2008) There are several types of both synthetic and natural polymers that can be fabricated into hydrogels, films, foams, hydrocolloids and beads such as cellulose, chitin, chitosan, collagen, gelatin and alginate. These materials can enhance the healing process and also used as drug carriers or therapeutic agents (Cardona *et al.,* 1996;; Suzuki *et al.*, 1998). Gelatin is a well-characterized protein fragment that can be hydrolyzed from collagens (Olsen, D., *et al*, 2003; Young, S., *et al* 2005). Gelatin is the protein consisting of 19 amino acid bonded by peptide bond. The composition and biological properties of gelatin can be identified to its precursor. Gelatin is popular in pharmaceutical and biomedical fields because its excellent biological activities and relative low cost (Young, *et al* 2005; Olsen, *et al*, 2003; Vlierberghe, *et al* 2008). Gelatin has also been used in medicine as the substrate for the drug delivery or other therapeutic agents, (Konishi, *et al*, 2005, Fukunaka, *et al*, 2002; Hori, *et al*, 2002) and wound dressing materials (Choi and Regenstein, 2000, Rattanaruengsrikul, *et al*, 2009). On the other hand, gelatin showed poor mechanical properties and it is easy to break when it dried or wet conditions. Therefore, gelatin hydrogels need to be crosslink for long-term applications (Venien and Levieux, 2005). There are various types of crosslinking agent used to ceosslink gelatin such as glutaraldehyde (Vandervoort,. and Ludwig, 2004; Zhang, *et al*, 2006; Ulubayram, *et al*, 2001; Miyashi, *et al*, 2005 and Huang, *et al*, 2006), genipin (Liu, *et al,* 2006), EDC/*N*-hydroxysuccinimide (NHS) (Liu, J., *et*

al, 2007), HDMI (Li, *et al*, 2005), EDC (Li, *et al*, 2006)). Glutaraldehyde is commonly used because of its relative low cost.

The use of hydrogels as wound dressings received much attention due to its toxicity, non-carcinogenic, and high biocompatibility. Furthermore, the use of hydrogels has the potential properties including its biocompatibility, high water content capacity, non-irritant and biodegradability. The *Eupatorium adenophorum* Spreng (Crofton weed) essential oils extracted from the plant *Eupatorium adenophorum* Spreng that are rich sources of biological active compounds. This family has been used in traditional medicine as antimicrobial, blood coagulant and anti-imflammatory activities (Rai and E. Sharma, 1994; Ansari, et al., 1983). Recently, *E. adenophorum* has been demonstrated to exhibit an antibacterial and antifungal effect with different strains of bacteria and fungi (Bhattarai and Shrestha, 2009).

In practical, most of bioactive compounds are poor water soluble that are the limitation for food industry or pharmacy applications. Therefore, oil in water emulsions use as the carriers for the delivery of *E. adenophorum* essential oil into the application of food, cosmetic and drug. In this work, we used pluronic F68® as a surfactant because pluronic F68® (a triblock ABA-type copolymer) was non-ionic, and non-toxic surfactant (Kibbe, 2000) and much attention as a surfactant in drug and cosmetic industries (Kabanov, Batrakova, & Alakhov, 2002).

In this paper, the oil-in-water emulsion-containing gelatin hydrogel pads have been fabricated for antibacterial wound dressings. The essential oil was first identified the chemical composition by using GC-MS analysis and determined their antibacterial in terms of minimum inhibitory concentration (MIC) and agar well diffusion method. The properties of the as loaded gelatin hydrogels in terms of gel fraction, swelling and weight loss behavior, water vapor transmission rate (WVTR) and tensile strength were also investigated to evaluate the usefulness of hydrogel.

5.3 Experimental

5.3.1 Materials

Gelatin powder, type A (obtained from porcine skin; 170-190 Bloom) was purchased from Fluka (Switzerland). Pluronic F68 was purchased from Sigma. It is a triblock copolymer based on poly(ethylene oxide)-block-poly(propylene oxide)-blockpoly(ethylene oxide) structure which is also typically expressed as PEOa–PPOb–PEOa, being a = 75 and b = 30. The *Eupatorium adenophorum* essential oil was obtained from Chulaborn Research Institute, Bangkok, Thailand. Glacial acetic acid was purchased from Mallinckrodt Chemicals (USA). Saturated glutaraldehyde (GTA) aqueous solution (5.6 M or 50% in water, used as the cross-linking agent) was purchased from Fluka (Switzerland). All other chemicals used were of analytical reagent grade and were used as received without further purification.

5.3.2 Plant Material and Extraction Procedure

The leaves of *Eupatorium adenophorum* were collected from Chiangmai province in northern Thailand. A voucher specimen was deposited at Laboratory of Natural Product, Chulabhorn Research Institute, Bangkok, Thailand. The fresh leaves were subjected to simple distillation apparatus for hydrodistillation. The distillation was performed for 2 hrs. The extract was dried in sodium sulfate anhydrous and stored in the refrigerator prior to analysis.

5.3.3 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed on ion-trap mass spectrometer (ITS-40, Finnigan mAT, USA) equiped with Varian 3400 GC (Varian, USA). The separation was accomplished on DB-5 capillary column (30m×0.25 mm. I.D., 0.25 μm film thickness, J&W Scientific USA). The temperature of an oven was increased from 40 °C to 200 °C at a rate of 4 °C per min. The injection port was set at 240 °C and split injection mode (split ratio 1:50) was used. The injection volume was 1μl with the sample concentration of 4.7 mg/ml. The essential oil was dissolved in dichloromethane. Helium (99.999%) was used as the carrier gas at the column head pressure of 15 psi. The ionization mode was electron ionization (EI) at 70 eV.

Identification of the constituents was identified retention index (homologous of series alkanes as the reference) and mass spectra library (NIST2005). The percent relative amount was calculated using GC-MS peak area without any correction.

5.3.4 Preparation of *Eupatorium Adenophorum* Emulsions

An oil-in-water (o/w) emulsion was prepared by homogenizing *E.adenophorum* essential oil and 15 % w/v Pluronic F68® aqueous solution (the volume ratio of essential oil to the pluronic solution = 5:5, 7:3, 8:2) under vigorous stirring for 3 min.

5.3.5 Zeta Potential Measurements

The essential oil emulsions (the volume ratio of essential oil to the pluronic solution = 5:5, 7:3, 8:2) were determined zeta potentials by a light-scattering instrument (Mastersizer 3000, Malvern Instruments, UK). It was measured four times for every sample in order to reach optimal measurement conditions.

5.3.6 Preparation of Neat Gelatin and emulsion-Loaded Gelatin Hydrogel Pads

Gelatin solution was prepared by 10 wt % of gelatin powder in aqueous solutions. The obtained solution was stirred at 40 °C in 1 h. The emulsion of *E.adephorum* 0-30% v/v was poured in the gelatin solution. Glutaraldehyde (GTA) aqueous solutions at 1μL/1 g of gelatin solution were mixed to cross-link gelatin hydrogels and stirred for 10 min. As-prepared gelatin containing emulsion of *E.adephorum* (12 mL) was then cast on a plastic petri dish, and dried at room temperature for 24 h. The gelatin hydrogel pads were kept in an oven at 40 °C for 24 h to complete the cross-linking reaction. The cross-linked gelatin hydrogels were soaked in 0.4 % w/v sodium metabisulfate solution for 24 h and then washed with distilled water.

5.3.7 Gel Fraction of The Neat and Emulsion-Loaded Gelatin Hydrogels

The gel fraction of the neat and as loaded hydrogels was extracted by water at 50 °C for 24 h to remove the non-crosslink parts to constant parts. The remained gels were kept at 50 °C in vacuum oven. The percentage of gel fraction was determined by using this equation:

$$\%\ Gel\ fraction = \frac{W_g}{W_o}\ x\ 100, \quad (1)$$

Where W_g is the weight of dried hydrogel after extraction and W_0 is the initial weight of dried hydrogels.

5.3.8 Swelling and Weight-Loss Behavior

The emulsion-loaded gelatin hydrogel in the circular shape (12 mm in diameter) were investigated by immersing the loaded hydrogels into the phosphate buffer (PBS) solution (pH 7.4) at 37 °C in different time points. Then, the swollen gel was weighted and the remained gels were then dried at 50 °C. The degree of swelling and weight loss were calculated as follows:

$$\%\ Swelling = \frac{W_s - W_i}{W_i}\ x\ 100, \quad (2)$$

$$\%\ Weight\ loss = \frac{W_i - W_d}{W_i}\ x\ 100\ , \quad (3)$$

where W_s is the weight of swollen hydrogel and W_i is the initial weight of dried hydrogel, and W_d was the weight of dry hydrogels after immersed in phosphate buffer (PBS) solution, respectively.

5.3.9 Water Vapor Transmission Rate of the emulsion-loaded hydrogels

The water vapor transmission rate (WVTR) of the emulsion-loaded hydrogels was measured by investigating the weight loss of 10 mL water in the bottle. The hydrogel specimen (15 mm) was placed on the mouth of the bottle and kept at 35 °C for 24 h. The water vapor transmission rate (WVTR) was determined by using this equation:

$$WVTR = \frac{W_i - W_t}{A \times 24} \times 10^6 \; g/m^2 h \,, \qquad (4)$$

where W_i is the initial weight of bottle, W_t is the weight of bottle after placed in oven, and A is the area of bottle mouth $(mm)^2$.

5.3.10 Loading Content and Release Characteristic of As-loaded emulsion From the emulsion-containing Gelatin Hydrogels

First, the actual amount of *E.adephorum* essential oil in the emulsion-loaded gelatin hydrogels was determined. The specimens were dissolved in 10 mL of 50 % nitric acid solution. The actual amount of *E.adephorum* essential oil within the hydrogels was then quantified by a UV spectrophotometer UV-1800 at wavelength 280 nm. In the release experiment, the samples of the emulsion-loaded gelatin hydrogel (2.8 cm in diameter) were immersed in the phosphate buffer solution (PBS) as the releasing medium at 37 °C. At each point in time, the PBS solution was removed 1 ml and was replaced with the fresh in the same amount. The released solution of *E.adephorum* essential oil was investigated by UV visible spectroscopy (UV-1800) at wavelength 280 nm. The obtained data were shown in the accumulative amount of *E.adephorum* essential oil divided by the actual weights of the emulsion-loaded gelatin hydrogel specimens.

5.3.11 Mechanical testing of the emulsion-loaded gelatin hydrogels

The tensile strength (MPa), and elongation at break (MPa) of the emulsion-loaded gelatin hydrogels were determined by using Lloyd LRX universal testing machine at the load cell 500 N, the crosshead speed 10 $mm \times min^{-1}$, and the gauge length 30 mm, respectively. The as-loaded gelatin hydrogels were cut into rectangular shapes (1 cm × 10 cm) and the thickness of the hydrogels were measured with a digital caliper. (Peacock digital thickness gauge Type SIS-6) The obtained data was the mean values of ten independent measurements.

5.3.12 Antimicrobial Evaluation of *E.adephorum* Essential oil

5.3.12.1 Minimum Inhibitory concentration (MIC)

The essential oil extracts of *E. adenophorum* were screened for their antibacterial activity, according to the minimum inhibitory concentration (MIC) was measured using the micro-dilution assay (National Committee for Clinical Laboratory Standards (United States), 1983) and the agar well diffusion method (Quiroga, 2001; Carron, 1987). Thirty one common bacterial strains were selected, which included Gram-positive species, *Bacillus cereus* ATCC 11778 (DMST 5040), *Staphylococcus aureus* ATCC 25923 (DMST 8840), *Staphylococcus aureus* DMST 20654 (MRSA), *Staphylococcus epidermidis* ATCC 12228 (DMST 15505), *Strephylococcus agalactiae* DMST 17129, *Strephylococcus pyogenes* DMST 17020, *Bacillus subtillis* ATCC 6633, *Enterococcus faecalis* ATCC 29212 (DMST 4736), *Listeria monocytogenes* DMST 17303 ,Gram-negative species, *Acinetobacter anitratus* DMST 4183, *Acinetobacter baumannii* ATCC 190066 (DMST 10437), *Acinetobacter calcoaceticus* ATCC 23055 (DMST 10436), *Acinetobacter lwoffii* ATCC 15309 (DMST 4229), *Burkholderia cepacia* ATCC 25416 (DMST 4205), *Escherichia coli* ATCC 25922 (DMST 4212), *Pseudomonas aeruginasa* ATCC 27853 (DMST 4739), *Pseudomonas fluorescens* DMST 6034, *Salmononelia enteritidis* ATCC 17368, *Salmononella typhi* DMST 5784, *Shigella dysenteriae* DMST 15111, *Vibrio cholerae* O139 ATCC 51394, *Vibrio cholerae* non O1, nonO139 DMST 2873, *Klebsiella pneumonia* ATCC 27736, *Klebsiella oxytoca* DMST 16071, *Escherichia coli* O157.H7 DMST 12743, *Proterus mirabilis* DMST 8212, *Serratia marcescens* ATCC 8100, *Shigella flexneri* DMST 4423, *Shigella sonnei* (group D)DMST 2982, *Shigella boydii* DMST 7776 and fungi species, *Candida albican* ATCC 10231. Mueller-Hinton (MH) broth was sterile by autoclaving and the portions were dispersed in petri dishes (9 cm in diameter). Serial dilutions of *E. adenophorum* essential oil were prepared from stock concentration (10000 µg/ml) and mixed with melted Muller-Hinton agar. The mixed solutions were immediately poured into Petri dishes (90 mm in diameter) after vortexing. The plates were spot inoculated with 10 µl (10^4 cells) of each microbial strain and incubated at 37 °C overnight. After incubated in 24 hr, MIC value is the lowest concentration that

can inhibit any visible microbial growth. *Staphylococcus aureus* and *Escherichia coli* were used as standard reference strains.

5.3.12.2 Agar-well diffusion assay

Only the bacterial strains that showed antibacterial activity from the minimum inhibitory concentration (MIC) assay were tested for agar-well diffusion assay. The inhibition zone of the essential oil extracts was determined against the following bacteria: *Acinetobacter calcoaceticus*, *Escherichia coli*, *Pseudomonas aeruginasa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Strephylococcus agalactiae*, and *Listeria monocytogenes*.

Bacteria were subcultured to nutrient agar and adjusted to 0.5 McFarland standard. The agar-well diffusion assay, *E. adenophorum* essential oils were poured into wells (5 mm) punched in the agar plates. The inoculums (about 10^4 CFU/ml) were uniformly and aseptically spread on the agar plate with a cotton swab and allow to solidify in a sterile workstation. Each well was aseptically filled up with 5,10,15,20 µl of *E. adenophorum* essential oil extracts. Agar plates were incubated at 37 °C overnight. DMSO, butanol and diethyl ether were introduced as controls. Antibiotics of chloramphenicol (30 µg), ampicillin (10 µg), penicillin G (10 units), kanamycin (30 µg), streptomycin (10 µg), and erythromycin (15 µg) were also used as positive controls. The inhibition zone was measured vertically and horizontally by using vernier caliper and the values expressed in average.

5.3.13 Antimicrobial Evaluation of the Emulsion-Loaded Gelatin Hydrogels

5.3.12.1 Agar disk diffusion assay

Neat gelatin hydrogels and Pluronic surfactant-loaded gelatin hydrogel were provided as the control. Amplicilin and chloramphinicol were provided standard groups for *S. aureus* and *E.coli*, respectively. The specimens (15 cm in diameter) were plated on agar plates. After incubate overnight at 37 °C. The agar plates were photographed and measured the inhibition zone of each specimen.

5.4 Results and discussion

5.4.1 The *E. adephorum* Essential Oil Composition

Essential oils were identified by Mass-spectrometer and the results were listed in Table 5.1. The principal component of the essential oil were *p*-cymene (16.23%), bornyl acetate (11.84%), amorpha-4,7(11)-diene (10.51%), alpha-phellandrene (7.829%), beta-caryophyllene (4.741%), camphere (4.133%), beta-bisabolene (4.03%). Other important compounds were E-beta-farnesene (3.341 %), alpha-trans-bergamotene (2.957%), cis-cadin-4-en-7-ol (2.653%). Papa-Paul *et al.,* reported the major constituents were p-cymene (11.64%), alpha-phellandrene (5.7%), γ-curcumene (5.0%), 2-carene (5.0%), camphene (4.8%), and endo-bornyl acetate (4.4%).

5.4.2 Zeta Potential Measurements

Zeta potential was determined by measuring electrophoretic mobility of particle of *E. adenophorum* essential oil extracts in Pluronic solution. Particles that have high zeta potential are self stablizing due to their charge protect coagulant (Morrision & Ross, 2002). The measured zeta potentials of oil-in- water emulsion by varying the volume of ratio *E. adenophorum* essential oil to pluronic solution was determined. The zeta potential in the volume ratio of essential oil and surfactant 5:5, 7:3, 8:2 were -5.07, -17.2, and 42 mV, respectively. The result showed that the zeta potential of 8:2 emulsion was a higher value when compared to other ratios. It means the particle are small enough, a high zeta potential will confer stability. In this work, we choose the ratio of 8:2 emulsion for further experiments.

5.4.3 Gel Fraction of Hydrogels

In order to crosslink gelatin polymer network, the glutaraldehyde was used as a crosslinking agent. The effect of emulsion content on the gel fraction is given in Fig. 5.1. The gel fraction in the neat gelatin hydrogel was about 69.82 ± 6.23 % resulting in gelatin has high cross-linked density. At a given glutaraldehyde concentration, the gel fraction of the hydrogels decreased with an increase in the percentage of as-loaded emulsion. The gel fractions of the emulsion-loaded

hydrogels were in the range of ~47.12 - 69.82 % resulting in the emulsion reduces the crosslink reaction and consequently the gelatin process because oil molecule can penetrate to gelatin networks which prevent intermolecular recombination of gelatin molecules leading to low gel fraction. Likewise, water enhances the mobility of the polymer to cross-link to each other when adding emulsion into the gelatin hydrogels decrease water content in the system resulting in decreasing gel fraction.

5.4.4 Swelling and Weight Loss Behavior

Fig.5.2 a represents the swelling behavior in different time point of the hydrogel dressings. At given glutaraldehyde concentration, the swelling of the neat and emulsion-loaded gelatin hydrogels increased with the submersion time. At the same time, the swelling behavior increases when increase the emulsion concentration, what is due to a decrease of the crosslink density related to the increase of the emulsion content. It was found that, the emulsion-loaded hydrogels have the degree of swelling in the range of 591.02-864.75%, 682.17-915.92, 871.37-1204.75, 941.54-2374.21 at 5%, 10, 20%, 30% the emulsion-loaded gelatin hydrogels, respectively.

The weight loss of the hydrogels in PBS pH 7.4 solution with various emulsion loaded at different time intervals were studied (Fig. 5.2 b). It was found that the weight loss of the emulsion loaded hydrogels in the range of 8.96-25.40, 18.39-26.85, 25.17-32.10, 29.15-36.79 at 5%, 10%, 20%, and 30% of the emulsion loaded, respectively. This is showed that the weight loss of the drug-loaded hydrogels increases with increasing the emulsion concentration in time dependent because of the degree of cross-linking network in the loaded hydrogels.

5.4.5 Water wapor transmission rate (WVTR)

The hydrogel wound dressing need to be control transmissions and absorption as well as maintain a moist environment in the wound area and calculated in terms of water vapor transmission rate (Peppas, 2000) (Table 5.2). The WVTR of the hydrogel loaded emulsion were shown in the range of 152-172 $g/m^2/h$. It was well known that the WVTR depended on thickness of specimens however the

hydrogels should tailor-made at the suitable thickness for appropriate use with any the wound types. Trade mark wound dressings such as Biabrone and Omiderm showed a WVTR at 154 and 208 g/m^2/h, respectively. There are no exact WVTR values for the suitable wound dressing however, if the WVTR was so high, it will cause faster drying and produce scars. On the other hand, a lower WVTR values, it will be risk in bacteria to grow in the wound area and inhibit the healing process (Balakrishnan, et al. 2005). The as-prepared gelatin hydrogels showed a value close to the range appropriate for wound dressing that would be control moisture, provide a good diffusion of gases and can adsorb the exudates on the wound care.

5.4.6 Release Characteristics of *E. adenophorum* essential oil

The actual amount of *E. adenophorum* essential oil in the hydrogels need to be investigated in order to determine the release characteristics of the emulsion-loaded gelatin hydrogels Experimentally, the actual amounts of *E. adenophorum* essential oil was 96.21 ± 0.21 % (n=3). Fig. 5.3 showed the percentage of the amount of essential oil loaded in the hydrogels by using total immersion method. The 20 % and 30% emulsion-loaded hydrogels showed a gradual release in the amount of *E. adenophorum* essential oil released from these specimens. The initial period rather rapidly release than within the first period of submersion and the quite slowly release afterwards. The first period that the *E. adenophorum* essential oil released rapidly from the 20 %, and 30 % emulsion-containing gelatin hydrogels was 2 h with the value of ~ 45.23 % and ~ 61.45%, respectively. The maximum amount of *E. adenophorum* essential oil released from the 20 %, and 30 % emulsion-containing gelatin hydrogels for 72 h were ~68.68 and ~90.56 %, respectively. As expected, the maximum amount *E. adenophorum* essential oil released from these materials increased when higher the initial amount of *E. adenophorum* essential oil loaded in the hydrogels.

5.4.7 Mechanical Properties

The stress at maximum load (MPa), percentage of strain at yield, and Young's modulus was evaluated and the results are summarized in Fig. 5.4. The

young's modulus of the emulsion-loaded gelatin hydrogels was 1691.21-938.43 MPa, the stress at maximum load was in the range of 19.52-10.16 MPa, and the percentage strain at yield was 2.48-1.53 %. The emulsion-loaded gelatin hydrogels decreased Young's modulus, the stress at maximum load, % strain at yield when emulsion-loaded gelatin, the crosslink density the hydrogel was decreased.

5.4.8 Antibacterial Evaluation of *E. adenophorum* Essential Oil

The antibacterial activity of *E. adenophorum* essential oil was first determined based on the Minimum Inhibitory concentration (MIC) and the Agar-well diffusion method, respectively against the bacterial strains

5.4.8.1 Minimum Inhibitory concentration (MIC)

The antibacterial activity of the *E. adenophorum* essential oil extracts was investigated in term of MIC values that are the ways to define the lowest concentration of substances inhibited the growth of microorganism and the result are summarized in Table 5.3. The MIC values of essential oil extracts were 78.125 to 1250 µg/ml. The *E. adenophorum* essential oil extracts were active against eight bacterial species. The essential oil showed the highest inhibitory activity against the Gram positive *S. Epidermis* with a MIC value of 78.125 µg/ml, followed by *S. agalactiae, B. subtillis* with MIC value of 312.5 µg/ml. The essential oil of *E. adenophorum* showed the MIC values against Gram negative bacterial. *A. calcoaceticus* showed the MIC value of 312.5 µg/ml and *E.coli* (625 µg/ml). Moreover, this essential oil extract evaluated showed antifungal activity against *C.albicans* with MIC value 312.5 µg/ml. Moreover, the antibacterial activity of essential oil with Gram negative bacteria showed less activity. It is due possibly to Gram negative cell walls are more complex preventing the entry of inhibitors (Anonymous, 2011). The external membrane of Gram negative bacteria has the presence of a hydrophobic surface that blocks the penetration of hydrophobic essential oils. Kurade *et.al.*, 2010 observed that *E. adenophorum* essential oil extract can inhibit *Arthrobacter protophormiae, Escherichia coli, Micrococcus luteus, Rhodococcus rhodochrous,* and *Staphylococcus aureus.* Moreover, It has been also reported the antibacterial and antifungal effect of *E. adenophorum* against 12 strains of bacteria and fungal isolates (Bhattarai, *et al.*, 2005). *P*-Cymene and bornyl acetate

were the main components of *E. adenophorum* essential oil that have a strong antifungal activity (Magwa, 2006).

5.4.8.2 Agar-well diffusion assay

The *E. adenophorum* essential oil extracts was showed the antibacterial activity against seven bacteria. The diameter of zone of inhibition was ranging between 0.9-2.3 cm in the antibacterial activity of *E. adenophorum* essential oil extracts in 5,10,15,20 µl. The zone of inhibition increased with increasing the amount of the essential oil extracts. The strongest antibacterial activity was observed in the 20 µl of the essential oil extracts as indicated in Table 5.4. Among Gram positive bacteria, maximum activity was exhibited against *S. aureus* ATCC 25923 (zone of inhibition 2.4 cm of 20 µl of the essential oil extracts) followed by *S. epidermidis*, *S. agalactiae*, and *L. monocytogenes* (inhibition zone 2.17 cm, 1.93 cm, and 1.84 cm), respectively. In case of Gram negative bacteria, the essential oil extracts showed maximum activity against *A. calcoaceticus* (inhibition zone 2.13 cm) followed by *E. coli* (2.03 cm) and *P. aeroginosa* (1.23 cm).

5.4.9 Antibacterial Evaluation of Emulsion-Containing Gelatin Hydrogels

The emulsion-containing gelatin hydrogels were determined their antibacterial activity in order to use as functional wound dressing by using the disc diffusion method against 8 types of pathogens: *Acinetobacter calcoaceticus*, *Escherichia coli*, *Pseudomonas aeruginasa*, *Staphylococcus aureus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Strephylococcus agalactiae*, and *Listeria monocytogenes*.

5.4.9.1 Agar disk diffusion

Agar diffusion test was used to evaluate the antibacterial activity of the neat, Pluronic solution-containing gelatin hydrogels, and the emulsion-containing gelatin hydrogels by measuring the inhibition zone length. The length of inhibition zone around specimens reveals degree of sensibility of the microorganism. Table 5 summarizes the zone of inhibition for all of the materials investigated. The neat and pluronic-loaded gelatin hydrogel pads showed no inhibit all types of bacteria, whereas the antibacterial activity of the materials was more effective

against *A.calcoaceticus*, *S.agalactiae*, *S.epidermidis*, *S.aureus* ATCC *25923*, *S.pyrogenes*, and *E. coli*, respectively.

5.5 Conclusion

In the present contribution, the oil-in-water emulsion of *E. adenophorum* essential oil extracts was slowly to the gelatin solution and successfully prepared into antibacterial wound dressings. To improve the mechanical properties of the as-loaded gelatin hydrogels, the glutaraldehyde solution was used as chemical crosslink agents. The gel fraction of the neat and emulsion-loaded gelatin hydrogels was in the range 31.52-69.82 % and decreases with increasing the content of emulsion. It is due to the oil may reduce the crosslink reaction. The water retention and weight loss behavior of the neat and emulsion-loaded gelatin hydrogels in phosphate buffer solution increased with any extended period of time. The total cumulative of *E. adenophorum* released from the hydrogels increased with increase in the emulsion content. The antibacterial activity was determined using agar disk diffusion methods. The 10-30 % emulsion gelatin hydrogels demonstrated the antibacterial activities against the eight pathogenic bacteria. These wound dressing hydrogels with antibacterial activity play important role to be used in wound care.

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Table 5.1 Chemical composition of *E. adenophorum* essential oil

Essential oil identification	**RI**	**Identification method**	**% relative**
alpha-Pinene	930	MS, RI(1)	0.174073
Camphene	944	MS, RI(1)	4.133385
beta-Pinene	972	MS, RI(1)	0.217394
2-Carene	998	MS, RI(1)	3.582081
alpha-Phellandrene	1002	MS, RI(1)	7.829352
Alpha-Terpinene	1014	MS, RI(1)	0.031279
Limonene	1022	MS, RI(1)	16.23725
Limonene	1026	MS, RI(1)	1.146877
E-Ocimene	1046	MS, RI(1)	0.278054
alpha-Terpinolene	1086	MS, RI(1)	0.200607
p-Cymenene	1086	MS, RI(1)	0
Linalool	1096	MS, RI(1)	0.313367

1,7-Octadien-3-one, 2-methyl-6-methylene-	1116	MS, RI(2)	0.115014
cis-p-Menth-2-en-1-ol	1118	MS, RI(1)	0.040849
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	1132	MS, RI(3)	0.033059
Camphor	1139	MS, RI(1)	0.028037
3Z-Hexenyl isobutanoate	1143	MS, RI(1)	0.062836
Hexyl isobutanoate	1148	MS, RI(1)	0.016075
Nerol oxide	1153	MS, RI(1)	0.014651
Borneol	1162	MS, RI(1)	0.686129
p-Cymen-8-ol	1182	MS, RI(1)	0.034423
alpha-Terpineol	1188	MS, RI(1)	0.427551
isopropyl methyl methoxy benzene isomer	1228	MS	0.168122

Thymol methyl ether	1233	MS, RI(1)	0.12233
Carvacrol methyl ether	1242	MS, RI(1)	0.327623
2-Caren-10-al	1271	MS	0.019693
Bornyl acetate	1284	MS, RI(1)	11.84321
Thymol	1292	MS, RI(1)	0.038911
trans-Pinocarvyl acetate	1298	MS, RI(1)	0.133501
Myrtenyl acetate	1324	MS, RI(1)	0.204601
alpha-Cubebene	1349	MS, RI(1)	0.073967
Monoterpene acetate	1362	MS	0.93067
Nerol acetate	1364	MS, RI(1)	0
NID	1368		0.473343
alpha-Copaene	1375	MS, RI(1)	0.066513
beta-Bourbonene	1384	MS, RI(1)	0.039168
7-epi-Sesquithujene	1390	MS, RI(1)	0.293931

NID	1394		0.263423
alpha-cis-Bergamotene	1414	MS, RI(1)	0.079424
beta-Caryophyllene	1419	MS, RI(1)	4.741454
Alpha-trans-Bergamotene	1435	MS, RI(1)	2.957541
Z-beta-Farnesene	1443	MS, RI(1)	0.810792
alpha-Humulene	1453	MS, RI(1)	0.11276
E-beta-Farnesene	1456	MS, RI(1)	3.341396
trans-Cadina-1(6),4-diene	1477	MS, RI(1)	0.203632
Amorpha-4,7(11)-diene	1480	MS, RI(4)	10.51046
NID	1485		3.562388
Neryl isobutyrate	1490	MS, RI(1)	1.272529
NID	1495		2.099217
Bicyclogermacrene	1496	MS, RI(1)	0
beta-Bisabolene	1509	MS, RI(1)	4.030827

Myristicin	1519	MS, RI(1)	0.102301
beta-Sesquiphellandrene	1524	MS, RI(1)	2.010994
NID	1530		3.976711
Beta-Vetivenene	1532	MS, RI(1)	0
NID	1534		0
NID	1543		0.505986
NID	1549		0.525996
NID	1576		0.98789
Caryophyllene oxide	1583	MS, RI(1)	0.563464
NID	1613		0.110209
NID	1622		0.858601
NID	1625		0
cis-Cadin-4-en-7-ol	1634	MS, RI(1)	2.653843
epi-alpha-Cadinol	1640	MS, RI(1)	0.300278
alpha-Cadinol	1654	MS, RI(1)	0.14141

epi-alpha-Bisabolol	1683	MS, RI(1)	0
alpha-Bisabolol	1686	MS, RI(1)	0.604471
NID	1690		0.203355
Amorpha-4,7(11)-dien-8-one	1698	MS, RI(5)	0.723202
3-alpha-Acetoxyamorpha-4,7(11)-diene	1774	MS, RI(4)	0.014374
Acetoxy amorpha-diene derivative	1789	MS	0.037369
Amorph-4-en-3,8-dione	1799	MS, RI(6)	0.543
Muurol-4-en-3,8-dione	1816	MS	0.15175
NID	1838		0.075687
NID	1844		0.077605
NID	1873		0.090714
NID	1916		0.026376

NID	1943		0.294248
NID	1964		0.047591
NID	1978		0.048797

Table 5.2 Water vapor transmission rate of the emulsion-loaded gelatin hydrogels for 24 h

Emulsion-loaded gelatin hydrogel (% v/v of gelatin)	**Water vapor transmission rate ($g/m^2/h$)**
5%	171.19 ± 5.46
10%	166.08 ± 16.21
20%	169.77 ± 16.44
30%	158.60 ± 9.69

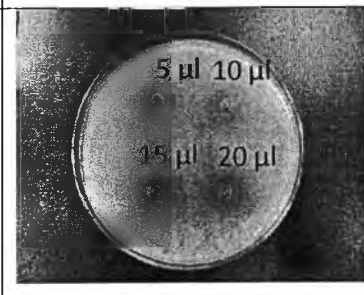
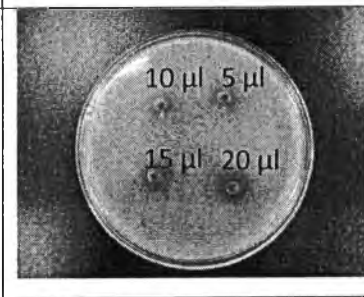
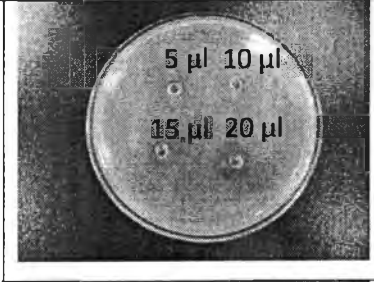
Table 5.3 Minimum inhibitory concentration (MIC) of *Eupatorium adenophorum* essential oils extracts

Microorganism	**MIC (µg/mL)**
Gram-positive bacteria	
Bacillus cereus ATCC 11778 (DMST 5040)	> 10,000
Staphylococcus aureus ATCC 25923 (DMST 8840)	1250
Staphylococcus aureus DMST 20654 (MRSA)	1250
Staphylococcus epidermidis ATCC 12228 (DMST 15505)	**78.125** [a]
Strephylococcus agalactiae DMST 17129	**312.5**
Strephylococcus pyogenes DMST 17020	2500
Enterococcus faecalis ATCC 29212 (DMST 4736)	> 10,000
Bacillus subtillis ATCC 6633	**312.5**
Listeria monocytogenes DMST 17303	> 10,000
Gram-negative bacteria	> 10,000
Acinetobacter anitratus DMST 4183	> 10,000
Acinetobacter baumannii ATCC 190066 (DMST 10437)	> 10,000
Acinetobacter calcoaceticus ATCC 23055 (DMST 10436)	**312.5**
Acinetobacter Iwoffii ATCC 15309 (DMST 4229)	> 10,000
Burkholderia cepacia ATCC 25416 (DMST 4205)	> 10,000
Vibrio cholerae non O1, nonO139 DMST 2873	> 10,000
Escherichia coli ATCC 25922 (DMST 4212)	625
Pseudomonas aeruginasa ATCC 27853 (DMST 4739)	2500
Pseudomonas fluorescens DMST 6034	> 10,000
Salmononelia enteritidis ATCC 17368	> 10,000
Salmononella typhi DMST 5784	> 10,000
Shigella dysenteriae DMST 15111	> 10,000
Vibrio cholerae O139 ATCC 51394	> 10,000
Klebsiella pneumonia ATCC 27736	> 10,000
Klebsiella oxytoca DMST 16071	> 10,000

Escherichia coli O157.H7 DMST 12743	> 10,000
Proterus mirabilis DMST 8212	> 10,000
Serratia marcescens ATCC 8100	> 10,000
Shigella flexneri DMST 4423	> 10,000
Shigella sonnei (group D)DMST 2982	> 10,000
Shigella boydii DMST 7776	> 10,000
Fungi	
Candida albican ATCC 10231	**312.5**

[a] Values boldly-written are considered very active (≤ 500 μg/ml)

Table 5.4 Antibacterial activity of the *E. adenophorum* essential oil extracts 5,1015,20 µl on seven types of bacteria stains. (Mean zone of inhibition (cm))

Type of bacteria	**5 µl**	**10 µl**	**15 µl**	**20 µl**	
A.calcoaceticus ATCC 23055 (DMST 10436)	1.80 ±0.02	1.88 ± 0.03	2.02 ± 0.08	2.13 ± 0.15	
E. coli ATCC 25922 (DMST 4212)	1.46 ± 0.04	1.6 ± 0.04	1.95 ± 0.02	2.03 ± 0.06	
P.aeruginasa ATCC 27853 (DMST 4739)	0.89 ± 0.02	1.00 ± 0.10	1.10 ± 0.10	1.23 ± 0.06	
S. aureus ATCC 25923 (DMST 8840)	1.84 ± 0.04	2.10 ± 0.10	2.13 ± 0.12	2.40 ± 0.10	

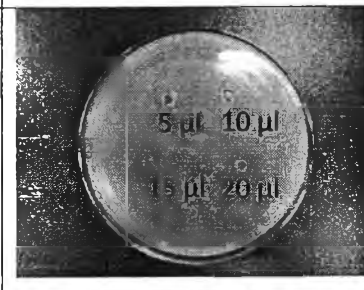
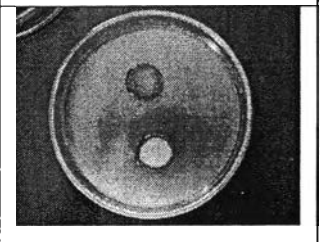
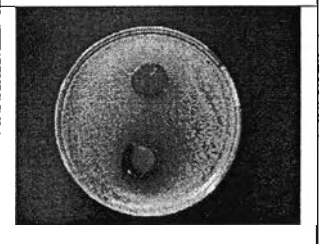
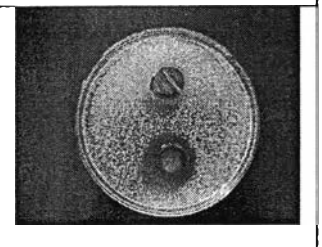
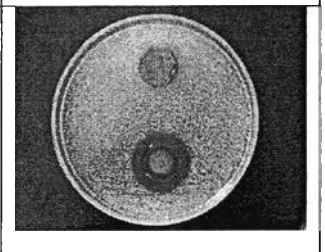
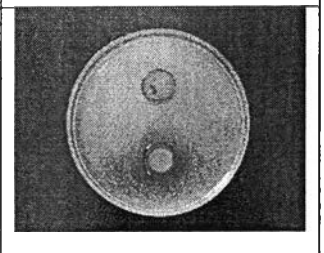
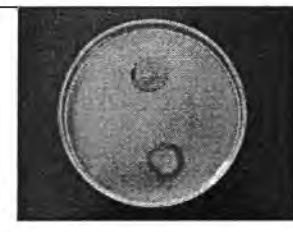
S.epidermidis *ATCC 12228* *(DMST 15505)*	1.66 ± 0.01	1.82 ± 0.02	1.91 ± 0.02	2.17 ± 0.20	
S.agalactiae *DMST 17129*	1.65 ± 0.03	1.76 ± 0.01	1.7 ± 0.01	1.93 ± 0.03	
L. monocytogenes *DMST 17303*	1.49 ± 0.01	1.60 ± 0.02	1.7 ± 0.02	1.84 ± 0.03	

Table 5.5 Average zone lengths of the inhibition zones (cm)

Type of bacteria	**20 % emulsion-loaded gelatin hydrogel**	**30 % emulsion-loaded gelatin hydrogel**	
A. calcoaceticus	3.26 ± 0.20	3.40 ± 0.10	
E.coli	2.366 ± 0.05	2.56 ± 0.03	
S.aureus ATCC 25923	2.55 ± 0.02	2.76 ± 0.05	
S.aureus (MRSA)	2.33 ± 0.05	2.77 ± 0.02	
S.epidermidis	2.87 ± 0.02	3.33 ± 0.15	

S.agalactiae	3.20 ± 0.2	3.45 ± 0.13	
L. monocytogenes	2.40 ± 0.02	2.90 ± 0.10	

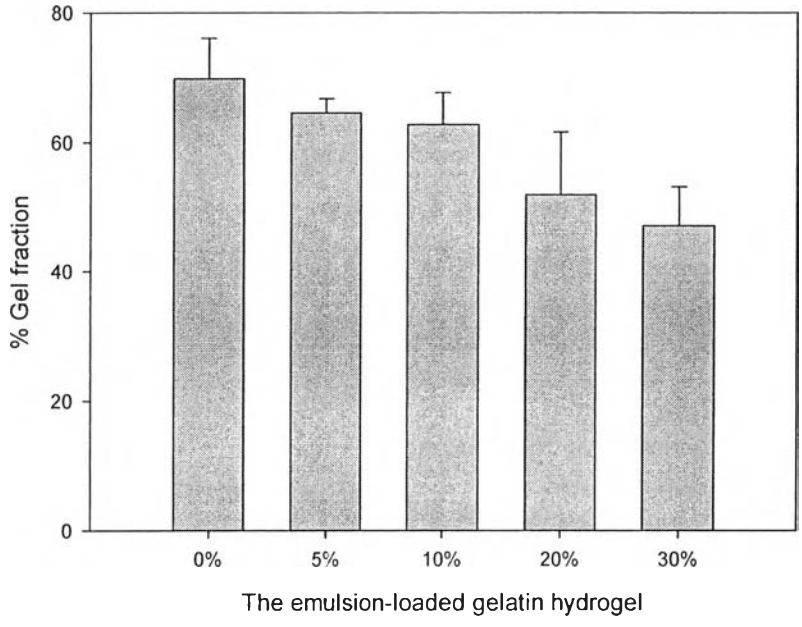
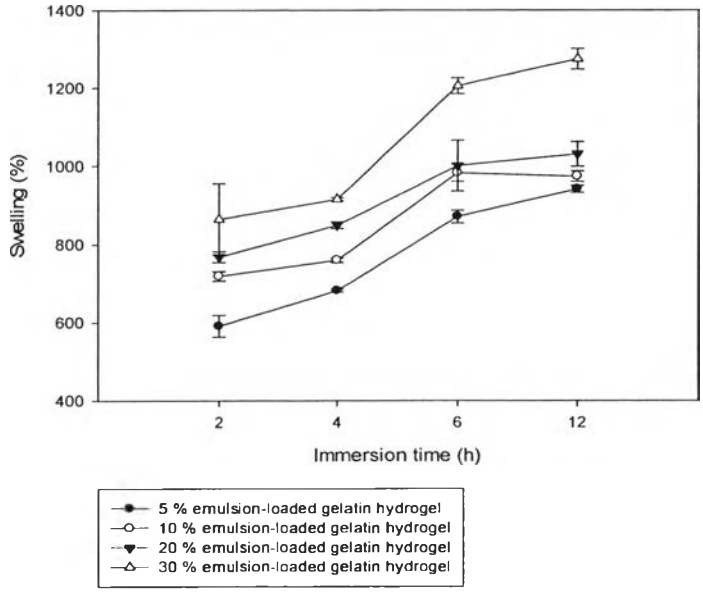


Figure 5.1 Gel fraction of the emulsion-loaded gelatin hydrogels at (0-30%v/v) 1μL/glutaraldehyde in 1 g of gelatin after having been extracted by water at 50 °C for 24 h (n=3).



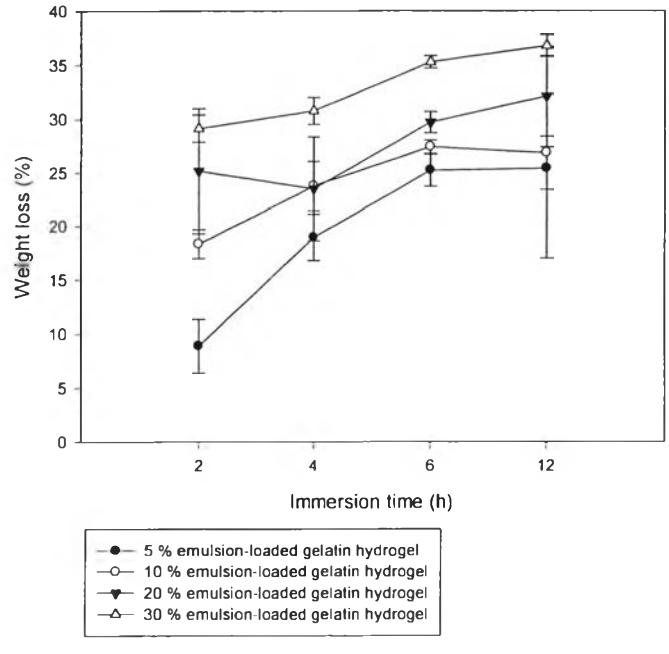


Figure 5.2 Degree of swelling and weight loss behavior of the emulsion-loaded gelatin hydrogels in phosphate buffer solution; PBS (pH 7.4) as a function of time.

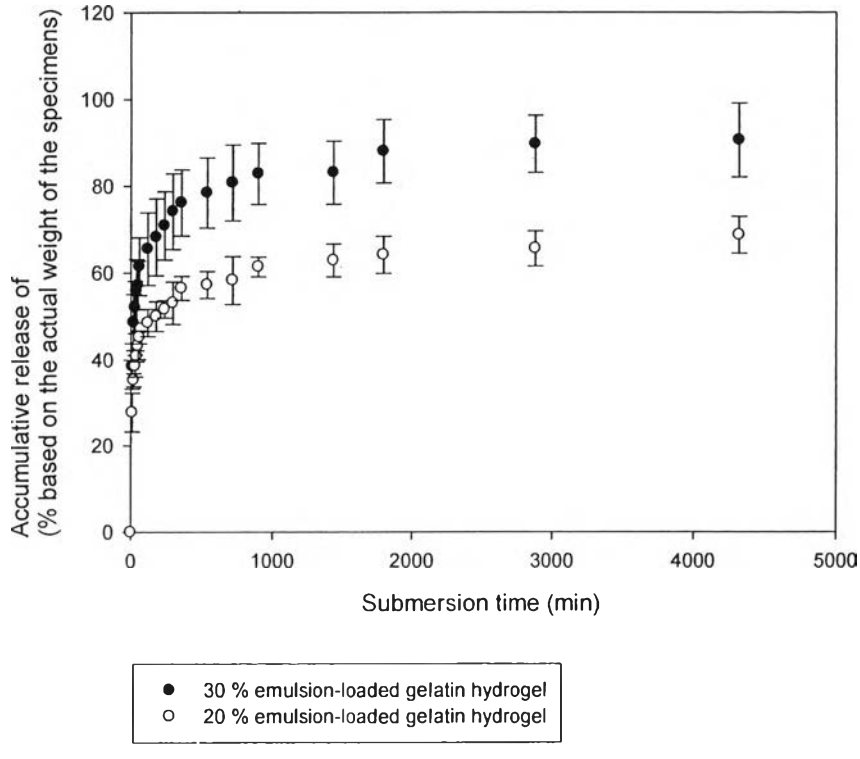
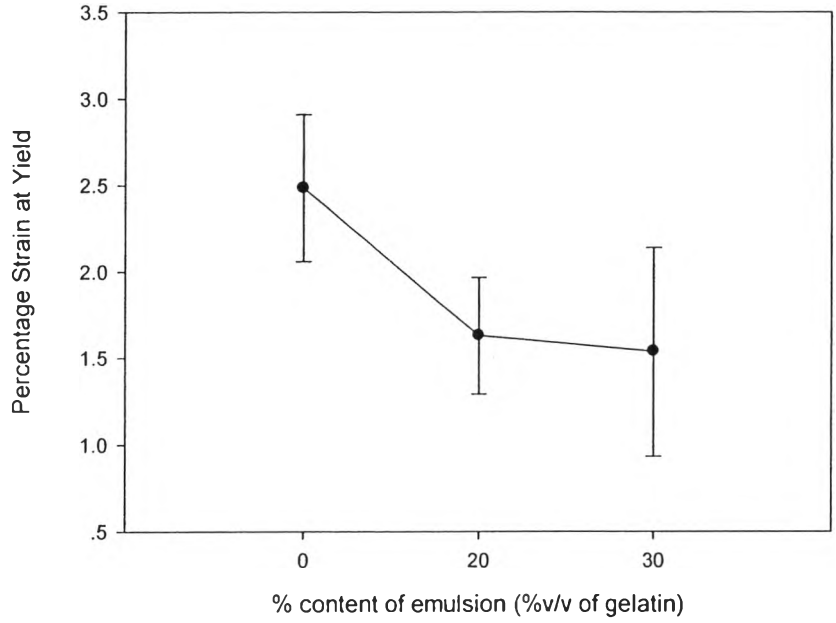


Fig. 5.3 Cumulative release profiles of *E. adenophorum* essential oil from the emulsion-loaded gelatin hydrogels reported as the percentage of the weight of *E. adenophorum* essential oil released divided by the actual weight of present in the specimens by total immersion method in the PBS releasing at the physiological temperature of 37 °C (n = 3).


3.5
3.0
2.5
2.0
1.5
1.0
.5
Percentage Strain at Yield
0
20
30
% content of emulsion (%v/v of gelatin)

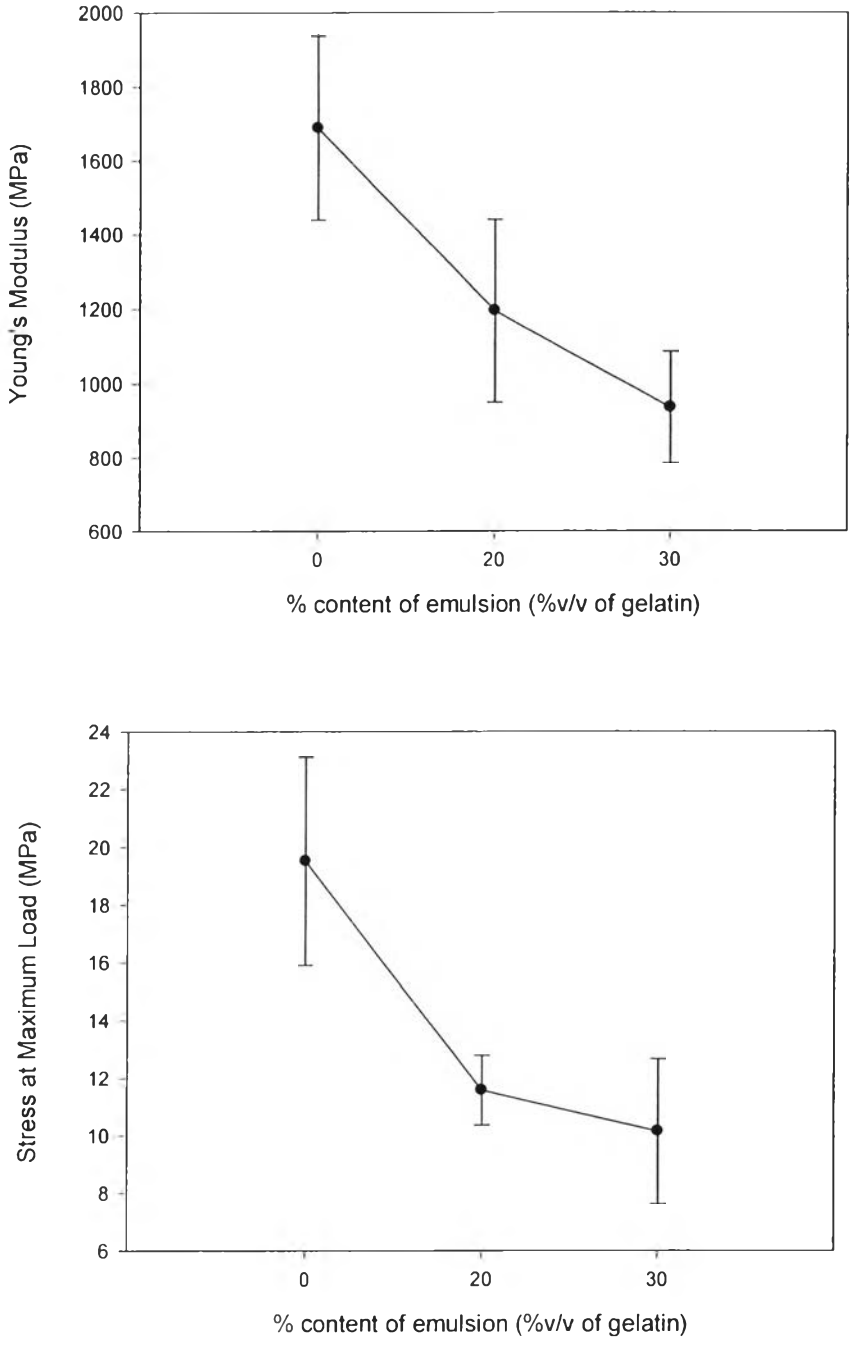


Fig 5.4 Mechanical integrity in terms of Young's modulus, yield strength, and elongation at yield of the neat and emulsion-loaded gelatin hydrogel pads (n=10).