Final Report

Project Title Development of a novel antimicrobial essential oil-based natural hydrogel wound dressing

By Dr. Piyachat Chuysinuan

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Abstract

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Project Period: 2 years

Abstract: Gelatin hydrogel was successfully prepared from a 10 wt-% gelatin solution and an essential oil, a herbal substance extracted from the *Eupatorium adenophorum* Spreng plant (Crofton weed), which is commonly used in traditional medicine due to its antimicrobial activity. The oil-in-water (o/w) emulsion *Eupatorium adenophorum* of the essential oil was prepared and used Pluronic F68® as a surfactant. The 10-30 %v/v emulsion was mixed with a gelatin solution and cast into a film. To improve the water resistance of the hydrogels, glutaraldehyde (GTA) was added to the emulsion-containing gelatin solution to crosslink the obtained gelatin hydrogels. These hydrogels were tested for their gel fraction, swelling and weight loss behavior. With an increase in the emulsion concentration of the emulsion-load in the hydrogels, the gel fraction decreased due to the crosslink density, while the swelling and weight loss behavior both increased in correlation with an increase in the emulsion content. The potential to use emulsion-containing gelatin hydrogels for the purpose of dressing a wound was assessed by investigating the release characteristics of the as-loaded hydrogels, and antimicrobial activity by using Agar disc diffusion methods. The results showed that *E. adenophorum* essential oil and the emulsion-load inhibited the growth of the test pathogens. Lastly, *E. adenophorum* essential oil was identified in terms of its composition by using GC-MS analysis and the determined antimicrobial activity of essential oil in terms of minimum inhibition concentration (MIC) used an

agar well diffusion method. The main chemical composition of the oil was made up from *p*-cymene (16.23%), bornyl acetate (11.84%), and amorpha-4,7(11)-diene (10.51%). All of the emulsion-loaded gelatin hydrogels were capable of inhibiting the growth of the tested pathogens, *S.agalactiae, A.calcoaceticus and S. epidermidis* and increased in correlation with the increase of the initial amount of emulsion in the hydrogels, which confirmed their potential application as antibacterial wound dressings.

Keywords: Wound dressing, essential oil, hydrogel, gelatin, silk protein

บทคัดย่อ

การพัฒนาวัสดุปิดแผลได้มีการเปลี่ยนจากวัสดุแบบเก่าซึ่งทำหน้าที่ปิดแผลไว้เพียงเพื่อป้องกันบาดแผลไม่ให้สัมผัสกับสิ่งแวดล้อมภายนอกไปเป็นวัสดุที่มีหน้าที่จัดการความชื้นและปลดปล่อยสารสำคัญออกมาสู่บริเวณแผลได้เพื่อให้วัสดุปิดแผลดังกล่าวมีประสิทธิภาพในการรักษาแผลมากขึ้น งานวิจัยนี้ได้ศึกษาการเตรียมวัสดุปิดแผลจากเจลาตินไฮโดรเจลที่ผสมสารสารสกัดจากพืชสาบหมาโดยเตรียมได้จากวิธีการ cast film สารสกัดจากพืชสาบหมาที่มีอยู่ในวัสดุปิดแผลมีคุณสมบัติในการยับยั้งเชื้อแบคทีเรีย ดังนั้นวัสดุปิดแผลที่ผสมสารสกัดจากพืชสาบหมาจึงมีคุณสมบัติยับยั้งการต้านเชื้อแบคทีเรียซึ่งเหมาะสมอย่างยิ่งในการนำไปประยุกต์ใช้เป็นวัสดุปิดแผลชนิดใหม่ช่วยส่งเสริมการรักษาแผลติดเชื้อในโรงพยาบาลได้ ในงานวิจัยนี้ได้สกัดพืชสาบหมาด้วยวิธีการกลั่นด้วยไอน้ำและวิเคราะห์สารสำคัญด้วยเครื่อง GC-MS จากนั้นทำการขึ้นรูปเจลาตินไฮโดรเจลผสมสารสกัดจากพืชสาบหมาและศึกษาลักษณะสัณฐานวิทยาของวัสดุปิดแผลที่เตรียมได้ด้วยกล้องจุลทรรศน์แบบส่องกราด คุณสมบัติทางกายภาพ ความสามารถในการบวมน้ำ คุณสมบัติเชิงกล และการปลดปล่อยสารำคัญออกจากวัสดุปิดแผลโดยใช้วิธี Total immersion method รวมถึงความสามารถในการยับยั้งเชื้อแบคทีเรียของสารสกัดพืชสาบหมาและวัสดุปิดแผลดังกล่าวอีกด้วย

การสกัดน้ำมันหอมระเหยจากใบสาบหมา (Eupatorium adenophorum Spreng) โดยการกลั่นด้วยไอน้ำทางตรงและเมื่อนำน้ำมันหอมระเหยที่ได้ไปวิเคราะห์หาองค์ประกอบด้วยเทคนิค GC/MS พบว่าน้ำมันหอมระเหยจากใบสาบหมา มีองค์ประกอบหลักคือ *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 %), E-beta-farnesene (3.341 %), alpha-trans-bergamotene (2.957 %), cis-cadin-4-en-7-ol (2.653 %) การสกัดเซริซินจากรังไหมด้วยวิธีทำแห้งเยือกแข็ง (lyophilization) และทำการศึกษาโครงสร้างทุติยภูมิของผงเซริซินที่เตรียมได้ด้วยเครื่องฟลูเรียทรานสฟอร์ม อินฟาเรด สเปกโทรโฟโตมิเตอร์ (Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy, ATR FT-IR) จะเห็นได้ว่าเส้นสเปกตรัมของผงเซริซินที่เตรียมได้โดยการทำแห้งเป็นผงของสารละลายเซริซินโดยวิธีการทำแห้งแบบเยือกแข็งจะแสดงพีกที่ตำแหน่งเลขคลื่น (wave number) เท่ากับ 1658 cm^{-1}, 1540 cm^{-1} และ 1240 cm^{-1} ซึ่งเป็นตำแหน่งพีกของหมู่ amide I, amide II และ amide III ตามลำดับ จากการศึกษาสมบัติทางกายภาพของแผ่นเจลาตินไฮโดรเจลที่ผสมสารสกัดจากน้ำมันหอมระเหยพบว่ารูปสัณฐานวิทยาของแผ่นเจลาตินไฮโดรเจลที่ขึ้นรูปด้วยเทคนิค solvent casting ด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด (Scanning Electron Microscope) จะพบว่าเจลาตินไฮโดรเจลที่ผสมอิมัลชั่น 10% ถึง 30% มีพื้นผิวที่เรียบ ไม่พบการแยกเฟสมีความเข้ากันได้ดี จะเห็นว่าสารละลายอิมัลชั่นสามารถกระจายตัวได้ดีในเจลาตินไฮโดรเจล และการปลดปล่อยของสารละลายอิมัลชั่นจากเจลาตินไฮโดรเจลแสดงในจะพบว่าที่เวลาต่างๆ การปลดปล่อยของสารละลายอิมัลชั่นเพิ่มขึ้นอย่างสม่ำเสมอและเริ่มคงที่ที่เวลา 500 นาที และเมื่อเพิ่มปริมาณน้ำมันหอมระเหยจะพบว่าร้อยละการปลดปล่อยมีค่ามากขึ้น จากการศึกษาฤทธิ์การยับยั้งเชื้อแบคทีเรียของสารสกัดสาบหมาพบว่าสามารถยับยั้งเชื้อได้ 2 ชนิด คือ *S. aureus* และ *S. Epidermidis* ได้ดี

1. Abstract:

Gelatin hydrogel was successfully prepared from a 10 wt-% gelatin solution and an essential oil, a herbal substance extracted from the *Eupatorium adenophorum* Spreng plant (Crofton weed), which is commonly used in traditional medicine due to its antimicrobial activity. The oil-in-water (o/w) emulsion *Eupatorium adenophorum* of the essential oil was prepared and used Pluronic F68® as a surfactant. The 10-30 %v/v emulsion was mixed with a gelatin solution and cast into a film. To improve the water resistance of the hydrogels, glutaraldehyde (GTA) was added to the emulsion-containing gelatin solution to crosslink the obtained gelatin hydrogels. These hydrogels were tested for their gel fraction, swelling and weight loss behavior. With an increase in the emulsion concentration of the emulsion-load in the hydrogels, the gel fraction decreased due to the crosslink density, while the swelling and weight loss behavior both increased in correlation with an increase in the emulsion content. The potential to use emulsion-containing gelatin hydrogels for the purpose of dressing a wound was assessed by investigating the release characteristics of the as-loaded hydrogels, and antimicrobial activity by using Agar disc diffusion methods. The results showed that *E. adenophorum* essential oil and the emulsion-load inhibited the growth of the test pathogens. Lastly, *E. adenophorum* essential oil was identified in terms of its composition by using GC-MS analysis and the determined antimicrobial activity of essential oil in terms of minimum inhibition concentration (MIC) used an agar well diffusion method. The main chemical composition of the oil was made up from *p*-cymene (16.23%), bornyl acetate (11.84%), and amorpha-4,7(11)-diene (10.51%). All of the emulsion-loaded gelatin hydrogels were capable of inhibiting the growth of the tested pathogens, *S.agalactiae, A.calcoaceticus and S. epidermidis* and increased in correlation with the increase of the initial amount of emulsion in the hydrogels, which confirmed their potential application as antibacterial wound dressings.

2. Executive summary

This research work is focusing on the development a novel hydrogel wound dressing based on gelatin and silk sericin as a biomaterials. The hydrogel wound dressing is incorporated with essential oil (a substance from the plant *Eupatorium adenophorum* Spreng (Crofton weed) which has commonly been used in traditional medicine to heal wounds). The antibacterial activities of the novel wound dressings are tested by using agar disk diffusion assay. The thickness uniformity, swelling and weight loss behavior, gel fraction, water vapor transmission rate, morphology, mechanical properties and in vitro drug release are investigated. Biomedical properties of novel wound dressings like in direct cytotoxicity, cell attachment and proliferation based on dermal human fibroblast are also evaluated.

2.1 Introduction to the research

Wound healing process is a complex process that involves the simultaneous actuation of soluble mediators, blood cells, extracellular matrix (ECM) and parenchymal cells [1]. Each of the wound stages involved in the process is usually characterized by the generation of some characteristic tissues and/or secretions which may as well require some specific treatment. The use of wound dressings may enhance the body's own healing mechanism by creating a proper physiologic wound environment (moisture and permeability to oxygen, water vapor and carbon dioxide), by acting as a barrier for microorganisms and/or by releasing bioactive compounds to the wound site [2–4]. Wound management is important in providing optimum healing milieu for wound healing. Based on these requirements, biocompatible polymeric hydrogels are promising materials for used as wound dressing. The desirable wound dressing may serve among the purposes of to provide moisture and occlusion, protection from infections and contamination, and easy application and removal. The choice of the dressing material is critical since its interaction with the wound may significantly influence the healing process. Natural-based biodegradable and biocompatible materials are gaining increasing attention. It is well known that gelatin is a product of the structural and chemical degradation of collagen. Gelatin has been widely used in pharmaceutical and biomedical fields because its biological origin, biodegradability, hydrogel properties and commercial availability at a relatively low cost [5-7]. Gelatin has also been used in medicine as sealants for vascular prostheses, plasma expanders, ingredients in drug formulations, carriers for the delivery of drugs or other therapeutic substances, [8] and, in particular, as wound dressing materials. [9]

Silk sericin is a protein created by *Bombyx mori* (silkworms) in the production of silk which contains 18 amino acids including essential amino acid. Silk sericin is characterized by excellent biocompatibility and biodegradability which is widely employed in biomedical fields

[10]. Moreover, silk sericin was investigated for its antioxidant [11] and antityrosinase activities in order to be employed in cosmetic and dermatilogic fields [12-13]. *Tsubouchu et al.* showed that sericin enhances the attachment of primary cultured human skin fibroblasts and accelerate wound healing in rats by facilitating collagenization as well as good compatibility and biodegradation. It may be a better candidate for wound dressing applications.

Occasionally, drug-loaded wound dressings are used to treat wound locally such as anti-infections due to secondary infection or for pain control, especially in chronic wounds. Various wound care products are available in the wound care management market and they are targeted towards the treatment of both acute and chronic wound. Therefore, in recent year, natural remedies have become more attractive for wound management. In Thailand, there are several types of herbal substances acted as wound healing. There are widely used for antibacterial, anti-inflammatory effects and induction of cell proliferation and skin cell differentiation towards an intact skin barrier. From this point of view, such traditional medicinal plants can effectively serve as identification of highly active new lead structures.

Essential oils from several plants have been used for the first aid treatment of wounds, abscess and burns. Essential oil is well-known to improve the wound healing process and antibacterial properties in traditional medicines. These oils contain numerous constitutes that contribute to the characteristic odour and medicinal effects. The major chemical components that account for the pleasant aromatic odours are primarily terpenenes, monoterpenes and linalool. The presence and quality of the various components varies between oils and determines the individuality of the oil. The plant *Eupatorium adenophorum* Spreng (Crofton weed) belongs to the family Asteraceae (Compositae). A number of plants of this family are commonly used in folklore medicine as antimicrobial, antiseptic, blood coagulant, analgesic, antipyretic and enhancer of phenobarbitone induced sleep. [14-15]. Ethanolic leaf extract of *E. adenophorum* reported for the first time the anti-inflammatory property [16]. Recently, *E. adenophorum* has been demonstrated to exhibit an antibacterial and antifungal effect with different strains of bacteria and fungi [17].

The aim of this study is to develop a novel antimicrobial essential oil-incorporated wound dressing. 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. Physical properties of the wound dressing including morphology, tensile strength, water swelling ability, and water vapor transmission rate (WVTR) were characterized. The *in vitro* attachment and proliferation of human dermal fibroblasts cultured in materials were investigated. The ability of the hydrogel dressing to retain and

release the antimicrobial compounds contained within the essential oil was studied *in vitro* using the agar diffusion assay against a range of clinically-relevant wound pathogens. Preliminary comparison of the essential oil-loaded hydrogel wound dressing with commonly used silver dressings was also performed.

2.2 Literature review

Wound healing is a specific biological process involving the general phenomenon of tissue regeneration [18]. The entire process of wound healing is a complex and ordered cascade of events, which can be divided into four distinct, but overlapping, phases of hemostasis, inflammation, proliferation and maturation. The factors related to improve wound healing which are antimicrobial and antioxidant characteristics. Open wounds are particularly prone to infection, especially by bacteria, and also provide an entrypoint for systemic infections. Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins will be produced with concomitant killing of regenerating cells. Antibacterial and antifungal compounds in a traditional remedy may prevent this occurring and may underlie its use in treating wounds. Probably the best antimicrobial assay to use is the serial dilution assay in microtitre well plates to assess minimum inhibitory concentration (MIC), although sometimes zone of inhibition studies are used. This latter approach is less reliable since it depends on the ability of extract components to diffuse through agar gel. The role of antioxidants in removing products of inflammation has been noted above but they are also beneficial in wound healing for other reasons. Antioxidants counter the excess proteases and ROS often formed by neutrophil accumulation in the wound area and protect protease inhibitors from oxidative damage. Fibroblasts and other cells may be killed by excess ROS and skin lipids will be made less flexible so antioxidant substances will reduce the possibility of these adverse events occurring. Because of these several factors, overall antioxidant effects appear to be important in the successful treatment of wounds. Antioxidant activity may be tested in several ways but several in vitro tests are commonly used. The DPPH method detects free radical scavengers, and uses a reduction in intensity of the coloured free radical 2,2-diphenyl-1-picrylhydrazine (DPPH).

Generally, an effectual wound dressing should maintain a moist environment upon absorption of the wound exudates, protect the wound from secondary infection, reduce necrosis of the wound bed, provide adequate gaseous exchange, regulate and/or mediate the release of certain growth factors and cytokines, and also be elastic, biocompatible with tissues and blood, non-toxic and non-antigenic. In addition, an effectual wound dressing should promote a rapid healing of the wound and, once healed, the detachment of the dressing should not cause secondary trauma to the neo-tissues. Among the wound dressings, special attention have been

given to hydrogel wound dressings due to their unique properties which meet the essential requirements of ideal wound dressings [19]. Such needs, in addition to the general requirements for an effectual wound dressing, include non-irritating and non-adhering properties, immediate pain relief, ease of handling and replacing without compromising patients' comfort, transparency to allow easy monitoring of the wound bed, and facilitation of the migration and mitosis of epithelial cells [20-22]. Hydrogels are a three dimensional network of polymer chains that can hydrophilic and can be absorb large amounts of water or biological fluids without being dissolved. An ideal wound dressing material should protect the injury from infection, maintain the wetness and humidity at wound area, reduce the risk of bacterial infection and enhance the healing [23]. 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. Among the various natural polymeric materials, gelatin is a well-characterized protein fragment that can be hydrolyzed from collagens and are found in tendons, cartilages, bones, skin and connective tissues [24]. Gelatin, an animal protein, consist of 19 amino acids joined by peptide linkages and can be hydrolyzed by a variety of the proteolytic enzymes to yield its constituent amino acid or peptide components. Its composition and biological properties are almost identical to its precursors. Gelatin has been widely used in pharmaceutical and biomedical fields because its biological origin, biodegradability, hydrogel properties and commercial availability at a relatively low cost [25-26]. Gelatin has also been used in medicine as sealants for vascular prostheses, plasma expanders, ingredients in drug formulations, carriers for the delivery of drugs or other therapeutic substances, and, in particular, as wound dressing materials [27].

Recently, we have been exploring a biological protein for wound dreesings. Silks represent a new family of advanced biomaterials due to their unique properties. Silk consists of two types of proteins, silk fibroin and sericin. It is characterized by its high content of serine and 18 amino acids. Silk sericin is a biocompatible protein which contains 18 amino acids including eesential amino acids. The biological properties of silk sericin are anti-oxidant, bio-adhesive and bioactive agent. Moreover, they reported that silk sericin can promote attachment and proliferation of fibroblasts, osteoblasts and keratinocytes resulting in accelarated wound healing. *Tsubouchu et al.,* showed that sericin enhances the attachment of primary cultured human skin fibroblasts and accelerate wound healing in rats by facilitating collagenization as well as good compatibility and biodegradation. It may be a better candidate for wound dressing applications. Silk fibroin from *Bombyx mori* has excellent mechanical properties, biocompatibility,

hemocompatibility, slow degradability, therein presenting considerable utility for a number of human therapeutic interventions. In addition, silk fibroin is able to support epidermal cells and fibroblast attachment, spreading, and proliferation, thus promoting wound healing. Fibroin films loaded with aloe gel extract were recently applied in the treatment of streptozotocin-induced diabetic rats. Compared to aloe-free fibroin film, the blended film enhanced the attachment and the proliferation of skin fibroblasts. Moreover the wounds in diabetic rats presented a smaller area 7 days after wounding (when compared to untreated diabetic wounds) and fibroblast distribution and collagen fiber organization similar to wounds in normal rats. These results show that accelerated wound healing can be obtained by using blended fibroin/aloe gel films which may be applied in the treatment of diabetic non-healing skin ulcers.

Bacterial contamination of a wound seriously threatens healing. In burns infection is the major complication after the initial period of shock, and it is estimated that about 75% of the mortalities following burn injuries are related to infections rather than to osmotic shock and hypovolemia. The incorporation of bioactive compounds (namely anti-inflammatories, anti-microbials, anti-septics, supplements, etc.) into wound dressing materials can play an active role in the wound healing process (either directly or indirectly) to prevent and mitigate inflammation, as cleansing agents, to prevent bacterial infection and to accelerate tissue regeneration by stimulating healthy healing responses with the minimum final scar formation.

Recently, Thai herbal substance has evoked considerable interest as food and feed additive and as an alternative to antibacterial agents, antibiotics and synthetic antioxidants etc. Plants and their extracts are compatible with the current thinking on the future of health care, agriculture, and food and consumer opinion that most “thing natural” are better and safer. These herbal substances from the traditional medicine are widely used due to the medicinal plants were extracted that contain several substances with many biological activities including antibacterial, antioxidant, and wound healing. In Thailand, they are several types of herbal substances has been used as wound healing during its biological activities such as antifungal, anti-inflammatory and promote cell proliferation towards an intact skin barrier. Thus, traditional medicinal plant has recently attracted much attention.

Essential oils (or volatile oils) are complex mixtures of volatile constituents that are biosynthesized by aromatic plants. They can be synthesized in buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood, or bark, and stored in secretory cells, cavities, canals, epidermal cells, or glandular trichomes. Essential oils are obtained by hydrodistillation, steam distillation, or dry distillation of a plant or plant part, or by a suitable mechanical process without heating (e.g., for citrus fruits). Vacuum distillation; solvent extraction combined off-line with

distillation; simultaneous distillation-extraction; supercritical fluid extraction; microwave-assisted extraction and hydrodistillation. The components of the Essential oils include regular terpenoids (mono-, sesqui-, and diterpenes), terpenoids with irregular carbon skeletons (homoterpenes and norisoprenoids), products of the lipoxygenase pathway (oxylipins), volatile fatty acid derivatives, indoles, and phenolics, including methyl salicylate and aromatic compounds. Many components of essential oils have been screened for antimicrobial activity. Essential oils are mainly composed of terpenoids, in particular monoterpenes and sesquiterpenes. The highest antimicrobial properties have been established in terpenes, such as carvacrol, geraniol, menthol, and thymol [28]. Carvacrol, citrals, p-cymene, and thymol contribute to increased membrane permeability and the swelling of cellular membranes. Carvacrol and thymol are thought to disturb the outer membrane of Gram-negative bacteria, leading to the release of lipo-polysaccharides [29]. Furthermore, p-cymene was shown to enable carvacrol influx due to its permeabilizing activity, thus resulting in synergistic activity when both components are present in an essential oils [30]. Another component with a phenolic structure, eugenol, may react with proteins and thus prevent the activity of enzymes in bacterial cells [29].

3. Objective

3.1 To fabricate and develop biodegradable and biocompatible natural polymers as wound dressing containing essential oil as a model drug to promote wound healing process and antibacterial activity

3.2 To investigate the potential for use of the natural polymers as biomedical applications including water swelling ability, release characteristics of essential oil and antibacterial activities of wound dressings

3.3 To evaluate the *in vitro* with human dermal fibroblast cells (HDFa) in terms of the indirect cytotoxicity the attachment and proliferation

4 Research methodology

4.1 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.

4.2 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 hours. The oils were dried over sodium sulfate anhydrous and stored in the refrigerator prior to analysis.

4.3 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). Oven temperature was increased from 40 °C to 200 °C at a rate of 4 °C per min. The injection port was set at 240 ºC 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 based on 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

4.4 Preparation of essential oil Emulsions

An oil-in-water (o/w) emulsion is prepared by homogenizing essential oil and 15 % w/v Pluronic F68® aqueous solution (the volume ratio of the pluronic solution to the essential oil : 5:5, 7:3, 8:2) under vigorous stirring for 3-10 min.

4.5 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 measurement instrument (Mastersizer, Hydro QS-M, Malvern Instruments, Ltd., Worcestershire, UK). It was measured four times for every sample in order to reach optimal measurement conditions.

4.6 Preparation of Neat Gelatin/silk sericin and emulsion-Loaded Gelatin/silk sericin Hydrogel Pads

Silk sricin are extracted using a hifh temperature and pressure degummimg technique. In brief, the silk worms cocoons are cut into small pieces and then put into deionized water and autoclaved at 120 °C for 60 min.Gelatin powder is dissolved in distilled water to obtain 10 wt% solution, and is stirred at 40 °C in 1 h. The silk sericin is added in to gelatin solution and then the emulsion of 0-30% v/v are followed added. Further stirring is used to homogenize the solution. Glutaraldehyde (GTA) aqueous solutions are mixed to cross-link gelatin hydrogels and under mechanical stirring for 10 min. The as-prepared gelatin containing emulsion is then cast on a plastic petri dish, and drying at room temperature for 24 h. The gelatin hydrogel pads are kept in an oven at 40 ºC for 24 h to complete the cross-linking reaction. In order to remove the unreact glutaraldehyde, the cross-link gelatin hydrogels are immersed in 0.1 M glycine solution for 2 h and then washed several times with distilled water. The thickness of the hydrogel pads in their dry state is measured for further experiments.

4.7 Fourier Transform Infrared Spectroscopy

FTIR spectra were obtained by attenuated total reflectance fourier trans for infrared (ATR-FTIR) spectroscopy (perkin elmer universal). The silk sericin using 32 scans at a resolution of 4 cm^{-1} from 400 to 4000 cm^{-1}.

4.8 Gel Fraction of The Neat and the emulsion loaded hydrogels

The gel fraction of the neat and as loaded hydrogels is extracted by water at 50 ºC for 24 h to remove the un-crosslink part. The remained gels are kept at 50 ºC in vacuum oven to constant weight. The percentage of gel fraction is calculated by using this equation:

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

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

4.9 Swelling and Weight-Loss Behavior

The emulsion-loaded gelatin hydrogel in the circular shape (12 mm in diameter) was investigated by immersing the loaded hydrogels into the phosphate buffer (PBS) solution pH 7.4 at 37 ºC for different time. Then, water on the surface of the swollen gel was removed

with filler paper and immediately weighted. The remained gel was then dried to constant weight at 50 ºC. 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.

4.10 Water Vapor Transmission Rate of the emulsion-loaded hydrogels

The water vapor transmission rate (WVTR) of the emulsion-loaded hydrogels was measured by measuring the weight loss of a bottle which contains 10 mL of water. The bottle has a mouth with a diameter of 13 mm. A round piece of hydrogel (15 mm) was mounted on the mouth of the bottle as a cap, then placed in an oven at 35 ºC for 24 h. The water vapor transmission rate (WVTR) was determined by using the following this equation:

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

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

4.11 Morphology of the emulsion-loaded hydrogels

Scanning electron microscope (SEM, Quanta 250 microscope, Japan) was used to study the morphology of microcapsules neem seed oil-loaded microcapsules powders. The sample was coated with a thin layer of gold using a JEOL JFC-1100 sputtering device prior to observation

4.12 Thermal transitions of the emulsion-loaded hydrogels

Differential scanning calorimeter (DSC) measurements of the emulsion-loaded hydrogels (10 mg) that were detected with 204F1 in the temperature rage 25-220 ºC and rate 10ºC/min.

4.13 Mechanical testing of the emulsion loaded hydrogels.

The tensile strength and breaking elongation of hydrogels were measured with a Lloyd LRX universal testing machine at room temperature. The emulsion-loaded gelatin

hydrogel pads in their dry state were cut into rectangular shapes (10 m × 10 m). The load cell, the crosshead speed, and the gauge length of the specimens were 500 N, 10 mm×min^{-1}, and 30 mm, respectively. The thickness of the hydrogel was measured with digital caliper. (Peacock digital thickness gauge Type SIS-6) The obtained data was the mean values of ten independent measurements.

4.14 Loading Capacity 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. The results were reported as average value (n=3). In the release assay, disc specimens of the emulsion-loaded gelatin hydrogel pads (circular disc; 2.8 cm in diameter) in their dry state were placed in 20 mL of phosphate buffer solution as the releasing medium at the physiological temperature 37 ºC for various time intervals. At each time point, the medium was totally removed and an equal amount of the fresh medium was replaced. The amount of the released *E.adephorum* essential oil in the withdrawn medium (i.e., sample solution) was determined by UV visible spectroscopy (UV-1800) at wavelength 280 nm. The obtained data were shown in the accumulative amount of *E.adephorum* essential oil based on the actual weights of the emulsion-loaded gelatin hydrogel specimens. At each time point, the measurements were carried out in triplicate.

4.15 Antimicrobial Evaluation of *E.adephorum* Essential oil

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 determined using the micro-dilution assay [31] and the agar well diffusion method [32]. 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. The bacteria were grown in sterile Mueller-Hinton (MH) broth and were cultured at 37 °C, for 24 h before the assay. Mueller-Hinton (MH) broth was sterile by autoclaving and 25 ml portions were dispersed in petri dishes (9 cm in diameter). The turbidity of the culture was adjusted with sterile saline solution to match 0.5 McFarland standard. Serial dilutions of *E. adenophorum* essential oil ranging 39.06 µg/ml to 10000 µg/ml were prepared from stock concentration (10000 µg/ml) and added to the sterile melted Muller-Hinton agar with the final volume 20 ml. The mixed solutions were immediately pour into Petri dishes (90 mm in diameter) after vortexing. The plates were spot inoculated with 10 µl (10^4 cells) of each microbial strains and incubated at 37 ºC overnight. After 24 h of incubation, MIC was defined as the lowest concentration able to inhibit any visible microbial growth. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as standard reference strains.

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 antibacterial tests using agar-well diffusion assay, as described by Eloff et al., 1998 [33] was used to obtain zone of inhibition of the essential oil extracts against the following bacteria: *Acinetobacter calcoaceticus* ATCC 23055 (DMST 10436), *Escherichia coli* ATCC 25922 (DMST 4212), *Pseudomonas aeruginasa* ATCC 27853 (DMST 4739), *Staphylococcus aureus* ATCC 25923 (DMST 8840), *Staphylococcus epidermidis* ATCC 12228 (DMST 15505), *Strephylococcus agalactiae* DMST 17129, *Strephylococcus pyogenes* DMST 17020, and *Listeria monocytogenes* DMST 17303.

Bacteria were subcultured to nutrient agar and incubated overnight at 37 °C and adjusted to 0.5 McFarland standard turbidity. Small wells were cut in the agar plate using a cork borer (5 mm). The inoculums (about 10^4 CFU/ml) was uniformly and aseptically spread on the agar plate with a cotton swab. The plate was allowed to dry for 5 min in a sterile

workstation. Each well was aseptically filled up with 5,10,15,20 µl of *E. adenophorum* essential oil extracts. The agar plates were incubated at 37 ºC for 24 h. DMSO, butanol and diethyl ether were introduced as controls. Antibiotics of ampicillin (10 µg), streptomycin (10 µg), penicillin G (10 units), kanamycin (30 µg), chloramphenicol (30 µg) and erythromycin (15 µg) were also used as positive controls. The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the zone inhibition in centimeters. The absence of a zone inhibition was interpreted as the absence of activity. The experiments were tested in triplicate.

4.16 Antimicrobial Evaluation of the Emulsion-Loaded Gelatin Hydrogels

Agar disk diffusion assay

The US Clinical and Laboratory Standards Institute (CLSI) disc diffusion method was used to assess the antibacterial activity of the emulsion-loaded gelatin hydrogel pads. Neat gelatin hydrogels and Pluronic surfactant-loaded gelatin hydrogel were used as the control group. Their antibacterial activity was tested against *Acinetobacter calcoaceticus* ATCC 23055 (DMST 10436), *Escherichia coli* ATCC 25922 (DMST 4212), *Pseudomonas aeruginasa* ATCC 27853 (DMST 4739), *Staphylococcus aureus* ATCC 25923 (DMST 8840), *Staphylococcus aureus* DMST 20654 (MRSA), *Staphylococcus epidermidis* ATCC 12228 (DMST 15505), *Strephylococcus agalactiae* DMST 17129, and *Listeria monocytogenes* DMST 17303. Amplicilin and chloramphinicol were used as antibacterial drugs for *S. aureus* and *E.coli*, respectively. The specimens (circular discs of 15 mm in diameter) and the drugs were placed on DifcoTM Mueller-Hinton agar in a Petri dish and then incubated at 37 ºC for 24 h. Finally, the agar plate was photographed to evaluate the antibacterial activity of each specimen. If inhibitory concentrations were reached, there would be no growth of the microbes, which could be seen as clear zones around the disc specimens.

5. Results and discussion

5.1 The *E. adephorum* Essential Oil Composition

The identified of the essential oils were listed in the table 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%). In 2010, Kurade, et al., [34] reported that the main components in essential oil of *E.adenophorum* were 1-napthalenol (17.50%), α-bisabolol (9.53%), bornyl acetate (8.98%), β-bisabolene (6.16%), germacrene-D (5.74%) and α- phellandrene (3.85%).

Table 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	1143	MS, RI(1)	0.062836

isobutanoate			
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-	1789	MS	0.037369

diene derivative			
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

5.2 Zeta Potential Measurements

Zeta potential is calculated by measuring the electrophoretic mobility of particles of *E. adenophorum* essential oil extract in a Pluronic solution. Particles with high zeta potential are self-stabilizing, as their charge inhibits coalescence and enhances stability. The measured zeta potentials of oil-in-water emulsion, conducted by varying the volume ratios of *E. adenophorum* essential oil to the Pluronic solution, was determined. The zeta potentials in the volume ratios of essential oil and surfactant 5:5, 7:3, 8:2 were -5.07, -17.2, and 42 mV, respectively. The results showed that the zeta potential of 8:2 emulsion was a higher value when compared to other ratios. This means that if the particles are small enough, a high zeta potential will confer stability. In this work, we chose a ratio of 8:2 emulsion to be applied for further experiments.

5.3 Fourier Transform Infrared Spectroscopy

Silk sericin were detected by attenuated total reflectance fourier transform infrared spectroscopy, ATR FT-IR. In general, silk sericin showed characteristic peaks at 1658 cm^{-} (amide I), 1540 cm^{-1} (amide II), 1020 (C-O stretching). Pure neem seed oil spectra showed characteristic peaks at 2853 (C-H stretching) 1240 cm^{-1} (amide III).

5.4 Gel Fraction of Hydrogels

The reaction of gelatin with glutaraldehyde in an aqueous solution leads to the formation of an insoluble polymer network (gel). The effect of emulsion content on the gel fraction is given in Fig. 1. The gel fraction in the neat gelatin hydrogel was approximately 69.82 ± 6.23 % and is considered as being relatively high, suggesting that gelatin was almost completely cross-linked. At a given glutaraldehyde concentration, the gel fraction of the hydrogels decreased in correlation with an increase in the percentage of as-loaded emulsion that had been loaded into the gelatin solutions. The gel fractions of the emulsion-loaded hydrogels were in the range of ~47.12 - 69.82 %. Thus, the emulsion decreased the gel fraction of the hydrogels. The emulsion reduces the crosslinking reaction and, consequently, the gelatin process, due to oil molecules penetrating gelatin networks which prevents intermolecular recombination of gelatin molecules leading to low gel fraction. Likewise, the presence of water enhances the mobility of the rigid molecules of the polymer, allowing macroradicals to cross-link to each other when adding emulsion into the gelatin hydrogels as a decrease in water content in the system results in a decrease in gel fraction.

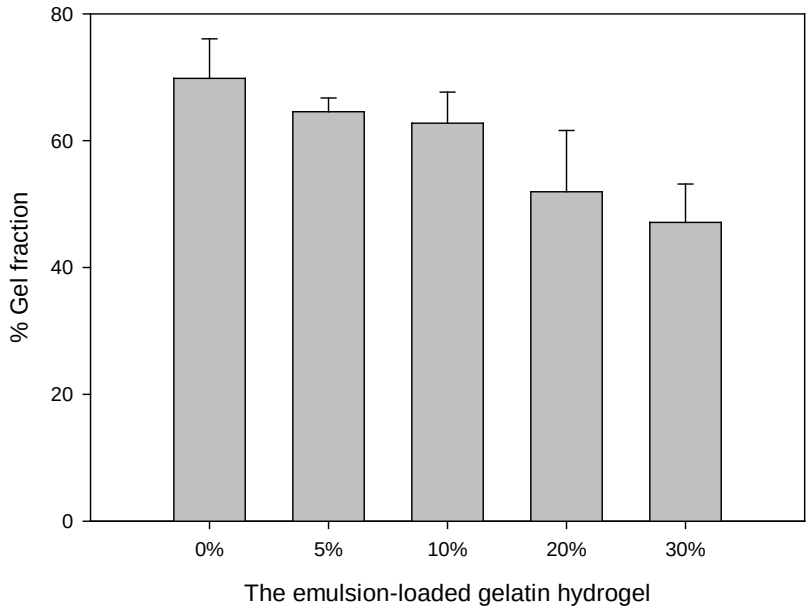


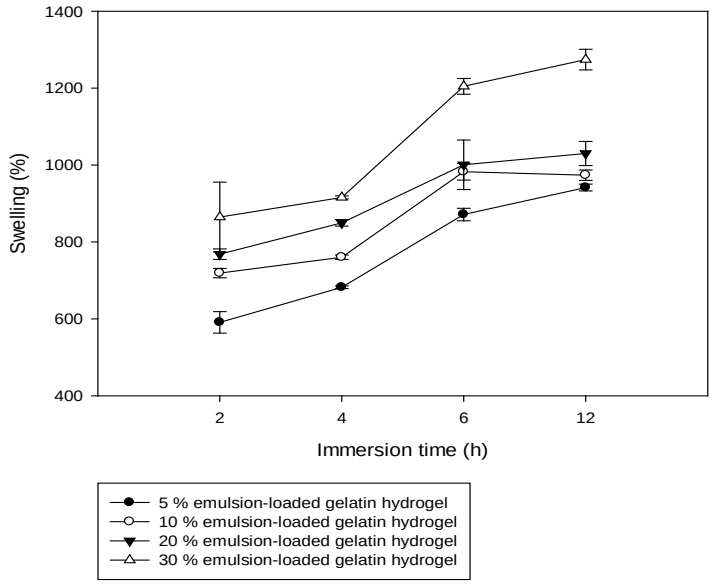
Figure 1 Gel fraction of the emulsion-loaded gelatin hydrogels at (0-30%v/v) 1µL/ glutaraldehyde in 1 g of gelatin after having been extracted by water at 50 ºC for 24 h (n=3).

5.5 Swelling and Weight Loss Behavior

Fig. 2 a represents the swelling behavior in different time point of the hydrogel dressings. At an given glutaraldehyde concentration, the swelling of the neat and emulsion-

loaded gelatin hydrogels increased with the submersion time. At the same time, it was observed that the swelling behavior increases with increasing 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. 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.



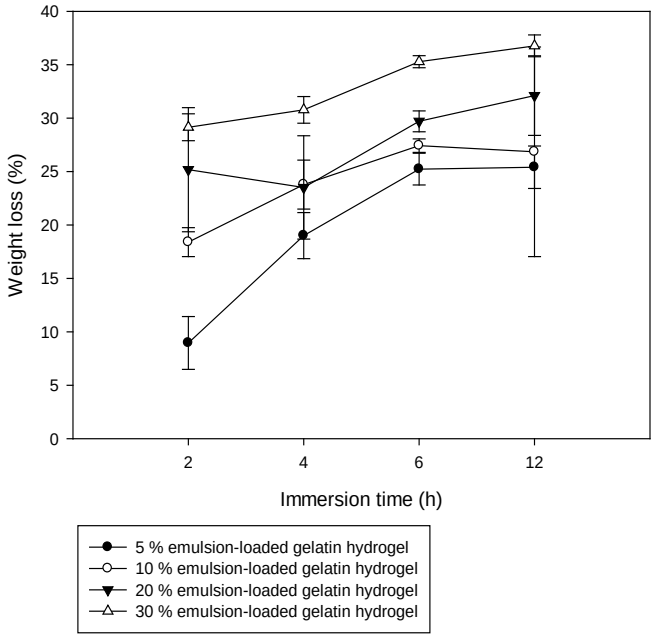


Figure 2 Degree of swelling and weight loss behavior of the emulsion-loaded gelatin hydrogels in phosphate buffer solution; PBS (pH 7.4) as a function of time**.**

5.6 Water wapor transmission rate (WVTR)

The hydrogel wound dressing need to be control absorption and transmission as well as maintain the high humidity in the wound area and calculated in terms of water vapor transmission rate [36] (Table 2). The WVTR of the hydrogel loaded emulsion were shown in the range of 152-172 g/m2/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. Wound dressings available in market such as Biabrone and Omiderm were found to have a WVTR of 154 and 208 g/m2/h, respectively. A higher value of WVTR causes a faster drying in the wound area and produce scars, whereas a lower WVTR accumulated exudates, which might retard the healing process and increase the risk of bacterial growth [37]. The as-prepared gelatin hydrogels showed a value close to the range appropriate for wound dressing that would be provide an adequate level of moisture to prevent excessive dehydration and build up the exudates on the wound area.

Table 2 Water vapor transmission rate of the emulsion-loaded gelatin hydrogels for 24 h

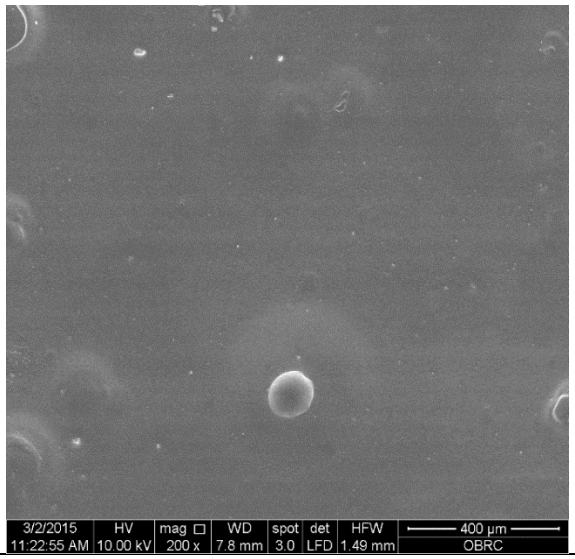
Emulsion-loaded gelatin	**Water vapor transmission rate**

hydrogel (% v/v of gelatin)	($g/m^2/h$)
5%	171.19 ± 5.46
10%	166.08 ± 16.21
20%	169.77 ± 16.44
30%	158.60 ± 9.69

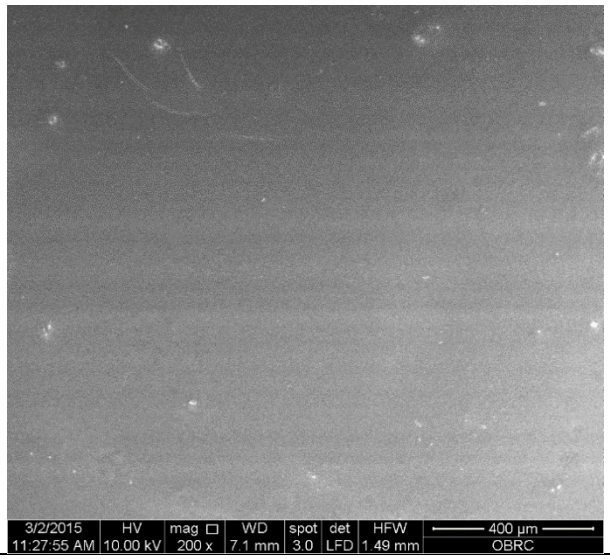
5.7 Morphology of the emulsion-loaded gelatin hydrogels

Morphology of the emulsion-loaded gelatin hydrogels (solvent casting) were detected by scanning electron microscope (SEM, Quanta 250 microscope, Japan). The SEM micrographs of hydrogels (10%-30%) showed smooth surface. In addition, hydrogels with emulsion no different when comparison hydrogel without emulsion.

(a)



(b)



(c)

(d)

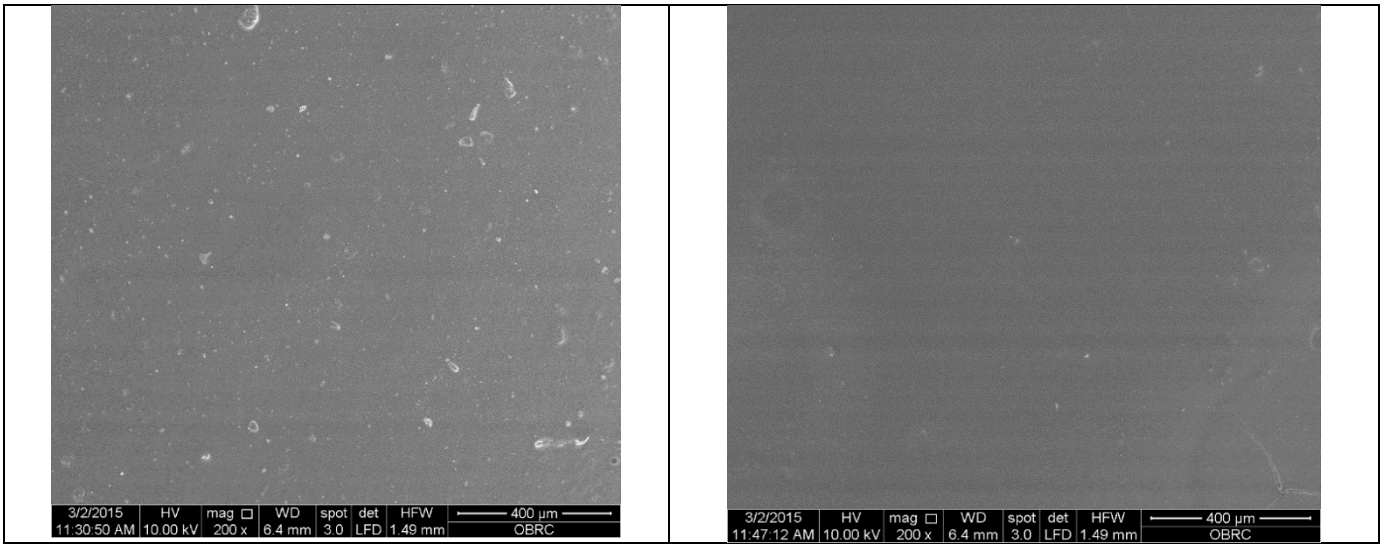


Figure 3 SEM pictures of the emulsion-loaded gelatin hydrogels (a) 0%, (b) 10 % (c) 20 % (d) 30 %

5.8 Thermo analysis of the emulsion-loaded gelatin hydrogels

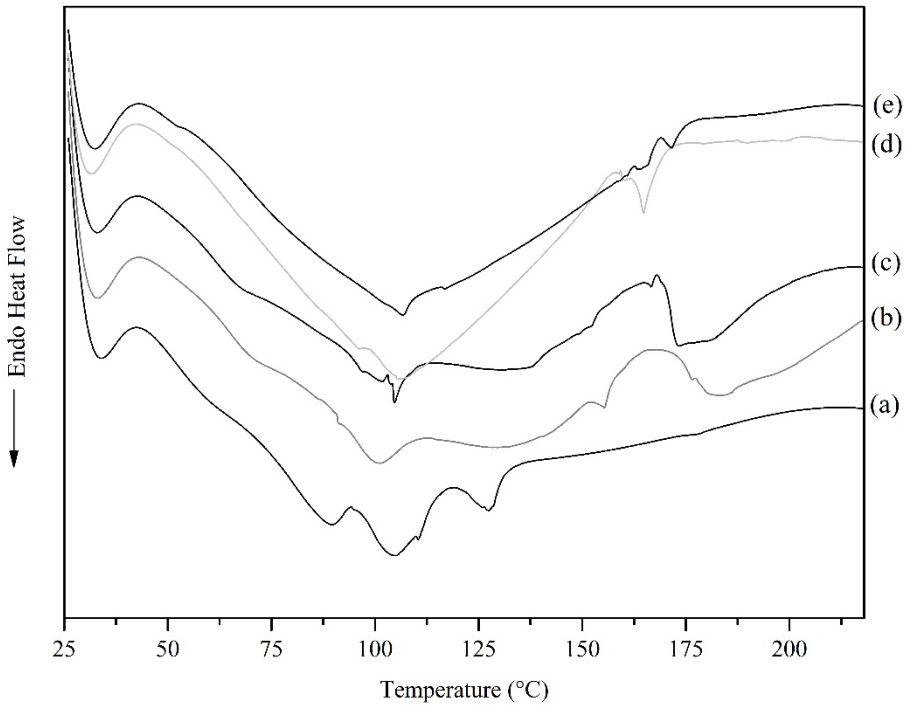
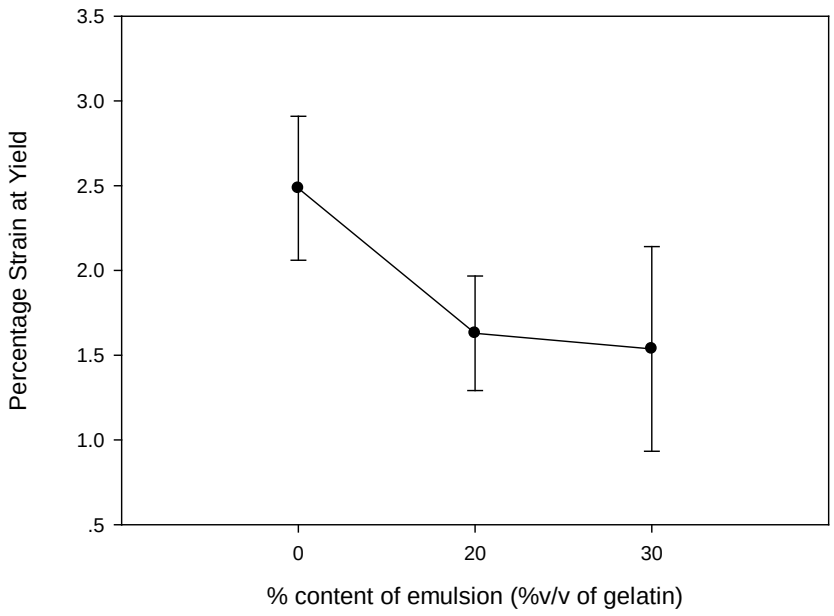
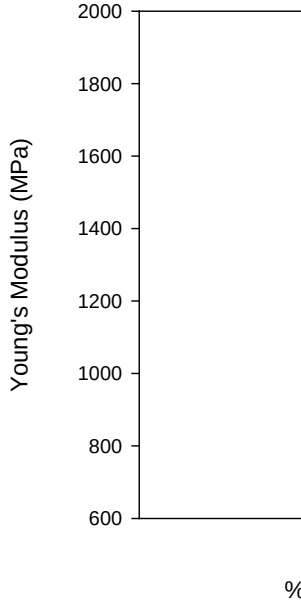


Figure 4 DSC of the emulsion-loaded gelatin hydrogels (a) 0%, (b) 5% (c) 10% (d) 15% (e) 20%

5.9 Mechanical Properties

To investigate the mechanical properties of the hydrogels, their stress at maximum load (MPa), percentage of strain at yield, and Young's modulus was evaluated. The results are summarized in Fig. 5. The young's modulus was in the range of 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 in the range of 2.48-1.53 %. The emulsion-loaded gelatin hydrogels decreased Young's modulus, the stress at maximum load, % strain at yield when emulsion was loaded with gelatin, the crosslink density the hydrogel was decreased.





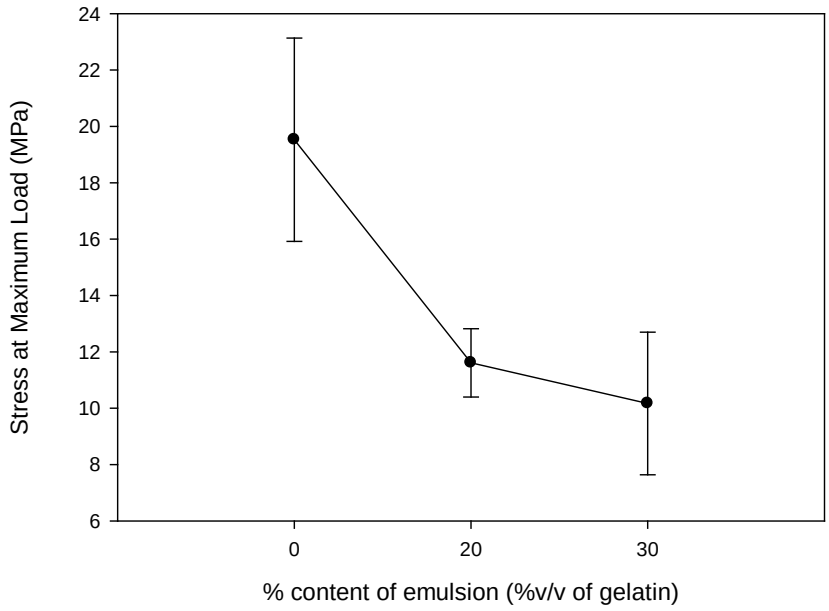


Figure. 5 Mechanical integrity in terms of Young's modulus, yield strength, and elongation at yield of the neat and emulsion-loaded gelatin hydrogel pads (n=10).

5.10 Release Characteristics of *E. adenophorum* essential oil

Prior to investigating the release characteristics of *E. adenophorum* essential oil from the emulsion-loaded gelatin hydrogel pads, the actual amount of *E. adenophorum* essential oil in the hydrogels needed to be determined. Experimentally, the actual amounts of *E. adenophorum* essential oil loaded in the hydrogels as determined by means of UV spectrophotometer in the PBS buffer were 96.21 ± 0.21 % (n=3). In the total immersion method (see Fig. 6), when reported as the percentage of the weight of *E. adenophorum* essential oil released divided by the actual weight of the specimens, the 20 % and 30% emulsion-loaded hydrogels showed a gradual increase in the amount of *E. adenophorum* essential oil released from these materials. The initial period rather rapidly release 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 with increasing the initial amount of *E. adenophorum* essential oil loaded in the hydrogels.

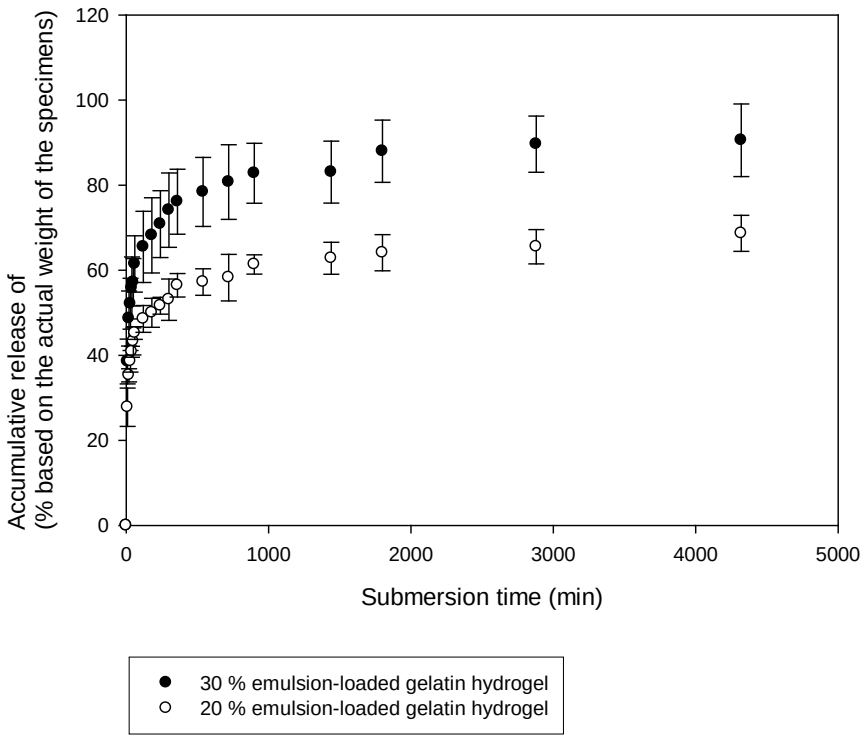


Figure.6 Cumulative release profiles of *E. adenophorum* essential oil from the emulsion-loaded gelatin hydrogels reported as the percentage of the weight of *E. adenophorum* essential oil released divided by the actual weight of present in the specimens by total immersion method in the PBS releasing at the physiological temperature of 37 °C (n = 3).

5.11 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

5.11.1 Minimum Inhibitory concentration (MIC)

The antibacterial activity of the *E. adenophorum* essential oil extracts is presented broad-spectrum activity against the bacterial strains used and the result are shown in table 3. The in vitro antimicrobial activity of the essential oil was expressed as minimum inhibitory concentrations (MIC). The MIC values obtained in this study from essential oil extracts tested ranged from 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* with MIC 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. According to these results, we can be concluded that *E. adenophorum* had a broad spectrum of antimicrobial activity. Less activity was observed with the Gram-negative bacteria in some cases probably due to their thick murein layer preventing the entry of inhibitors [38].The external membrane of Gram negative bacteria has the presence of a hydrophobic surface that blocks the penetration of hydrophobic essential oils [36] observed that *E. adenophorum* essential oil analyzed by Gas chromatography-mass spectrometry (GC-MS) inhibited the growth of *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 [39]. This result supported the antifungal activity against *C.albicans* with MIC value of 312.5 µg/ml of *E. adenophorum* essential oil.

5.11.2 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 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* and *P. aeroginosa* (Inhibition zone 2.03 cm and 1.23 cm), respectively.

5.12 Antibacterial Evaluation of Emulsion-Containing Gelatin Hydrogels

The potential for use of the emulsion-containing gelatin hydrogels as functional wound dressings was assessed by observing their antibacterial activity based on the disc diffusion method against 8 types of bacteria: Acinetobacter calcoaceticus ATCC 23055 (DMST 10436), Escherichia coli ATCC 25922 (DMST 4212), Pseudomonas aeruginasa ATCC 27853 (DMST

4739), Staphylococcus aureus ATCC 25923 (DMST 8840), Staphylococcus aureus DMST 20654 (MRSA), Staphylococcus epidermidis ATCC 12228 (DMST 15505), Strephylococcus agalactiae DMST 17129, and Listeria monocytogenes DMST 17303

5.12.1 Agar disk diffusion

Disc diffusion method 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 average lengths of the inhibition zones for all of the samples investigated. As for the neat and pluronic-loaded gelatin hydrogel pads, no inhibition zones were observed against all types of bacteria, a result that is in line with the nonbactericidal property of the neat and surfactant-loaded materials. On the other hand, inhibitory zones were obvious for all of the emulsion-containing specimens. Apperently, the lengths of the inhibition zones generally increased with increases in both the initial concentration of emulsion and 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.

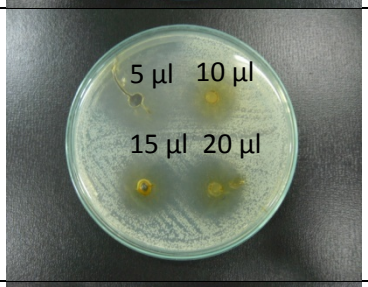
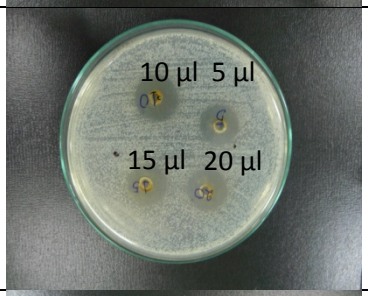
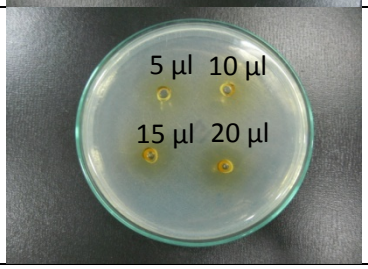
Table 3 Minimum inhibitory concentration (MIC) of *Eupatorium adenophorum* essential oils extracts

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 lwoffii 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 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 of essential oil extracts**	**10 µl of essential oil extracts**	**15 µl of essential oil extracts**	**20 µl of essential oil extracts**	
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	1.62 ± 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	

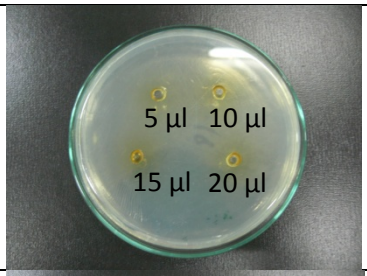
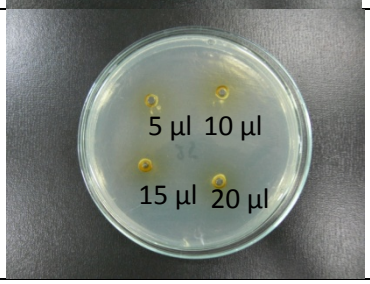
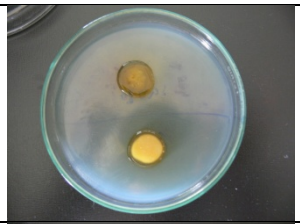
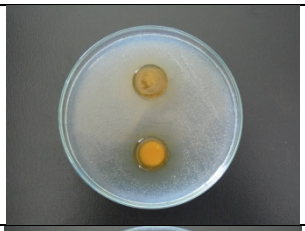
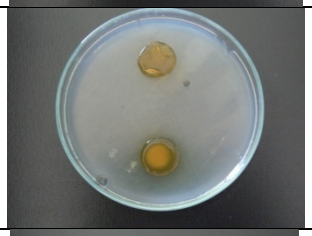
S.agalactiae DMST 17129	1.65 ± 0.03	1.76 ± 0.01	1.79 ± 0.01	1.93 ± 0.03	
L. monocytogenes DMST 17303	1.49 ± 0.01	1.60 ± 0.02	1.71 ± 0.02	1.84 ± 0.03	

Table 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 ± 0.20817	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	
S.pyrogenes	2.40 ± 0.02	2.90 ± 0.10	

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6. Conclusion

In the present contribution, the oil-in-water emulsion of *E. adenophorum* essential oil extracts and Pluronic F68® (emulsion at 10-30 % v/v) was added to the gelatin solution (10% w/v in water) and successfully prepared as antibacterial wound dressings. The neat and emulsion-loaded gelatin hydrogels were chemically cross-linked with glutaraldehyde to improve their mechanical integrity in the wet state. 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 crosslinking reaction. The swelling and weight loss behavior of the neat and emulsion-loaded gelatin hydrogels in phosphate buffer solution increased with the submersion time. The total cumulative amount of *E. adenophorum* released from the hydrogels was found to increase with an increase in the emulsion content. The potential for use of the emulsion-loaded gelatin hydrogels as wound dressings was assessed by antibacterial activity using agar disk diffusion methods. The results showed that the hydrogels were effective against the eight pathogens. The antibacterial activity of the emulsion-loaded hydrogels increases with increasing the emulsion content. This wound dressing hydrogel with antibacterial activity is highly potential to be used in tropical environment.

7. Appendix

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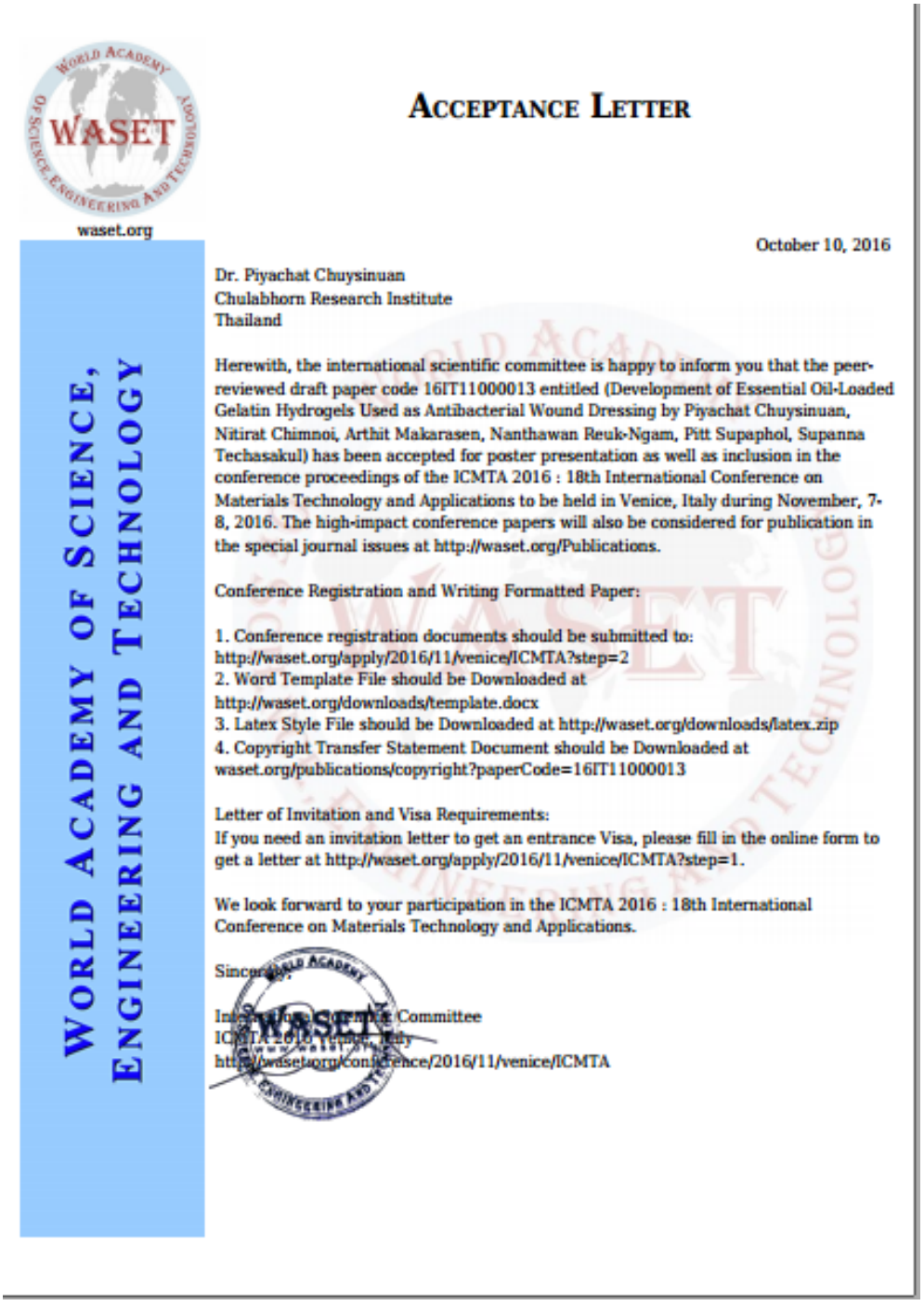
8. Output (Acknowledge the Thailand Research Fund)

8.1 International Journal Publication

8.2 Application

8.3 international conference



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October 10, 2016

Dr. Piyachat Chuysinuan
Chulabhorn Research Institute
Thailand

Herewith, the international scientific committee is happy to inform you that the peer-reviewed draft paper code 16IT11000013 entitled (Development of Essential Oil-Loaded Gelatin Hydrogels Used as Antibacterial Wound Dressing by Piyachat Chuysinuan, Nitirat Chimnoi, Arthit Makarasen, Nanthawan Reuk-Ngam, Pitt Supaphol, Supanna Techasakul) has been accepted for poster presentation as well as inclusion in the conference proceedings of the ICMTA 2016 : 18th International Conference on Materials Technology and Applications to be held in Venice, Italy during November, 7-8, 2016. The high-impact conference papers will also be considered for publication in the special journal issues at http://waset.org/Publications.

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