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Cornstarch-based wound dressing incorporated with hyaluronic acid and propolis: *In vitro* and *in vivo* studies

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ABSTRACT

The unique physicochemical and functional characteristics of starch-based biomaterials and wound dressings have been proposed for several biomedical applications. Film dressings of cornstarch/hyaluronic acid/ ethanolic extract of propolis (CS/HA/EEP) were prepared by solvent-casting and characterized by attenuated total reflectance/Fourier transform infrared spectroscopy, scanning electron microscopy, gas chromatography/mass spectrometry, light transmission, opacity measurements, EEP release, equilibrium swelling, and *in vitro* and *in vivo* evaluations. The CS/HA/0.5%EEP film dressing exhibited higher antibacterial activity against *Staphylococcus aureus* (2.08 ± 0.14 mm), *Escherichia coli* (2.64 ± 0.18 mm), and *Staphylococcus epidermidis* (1.02 ± 0.15 mm) in comparison with the CS, CS/HA, and CS/HA/0.25%EEP films. Also, it showed no cytotoxicity for the L929 fibroblast cells. This wound dressing could effectively accelerate the wound healing process at Wistar rats' skin excisions. These results indicate that enrichment of cornstarch wound dressings with HA and EEP can significantly enhance their potential efficacy as wound dressing material.

1. Introduction

Wound healing is the primary response to any tissue injuries (Sen et al., 2009). Utilizing wound dressing for wound healing has received lots of attention (Arockianathan, Sekar, Sankar, Kumaran, & Sastry, 2012; Kamoun, Kenawy, & Chen, 2017). An ideal wound dressing should exhibit specific properties including flexibility, durability, wound hydration/dehydration and suitable mechanical properties.

Starch is a biocompatible and biodegradable natural polymer. It is a low-cost polysaccharide with high availability and water affinity (Pal, Banthia, & Majumdar, 2006; Torres, Commeaux, & Troncoso, 2013). Starch is composed of amylose and amylopectin which relative amounts of each component vary according to its plant source. Amylose is a slightly branched polymer of α-D-glucose units linked by α-1,4-glycosidic bonds; amylopectin is a highly branched polymer consisting of α-D-glucose units linked by α-1,4-glycosidic bonds and α-D-glucose units by α-1,6-glycosidic bonds. The ratio of these two polymers is generally related to the botanic origin of starch (Luzi et al., 2018; Wilpiszewska, Antosik, & Spychaj, 2015). As an example, cornstarch has about 28 wt.% amylose as compared to cassava starch with 17 wt.%. Film-forming and mechanical properties are dependent on amylose to amylopectin ratio. In general, an increasing amount of amylose improves the above-mentioned properties (Forssell, Lahtinen, Lahelin, & Myllärinen, 2002; Raquez et al., 2008). Previous studies have reported that amylose content of the starch have direct relation with the strength and toughness of the dressings (Alcázar-Alay & Meireles, 2015). Cornstarch film dressing can provide local moist environment, good surface absorbance for wound exudate, and decrease of wound surface necrosis.

Cornstarch wound dressing doesn't exhibit antimicrobial features and should be enriched with antimicrobial agents for preventing wound infection (Lozano-Navarro et al., 2017; Naseri-Nosar & Ziora, 2018; Villanueva et al., 2016). Many antimicrobial agents such as copper (Borkow et al., 2010), silver (H. Chen, Lan et al., 2018), etc have been used for fortifying wound dressings. Fang et al. have synthesized antimicrobial starch-PVA film dressing by chemical crosslinking of starch, PVA and oligoguanidine (S-PHMG) (Fang et al., 2011). These films dressings exhibited antimicrobial activities against both *Escherichia coli* and *Staphylococcus aureus*. Propolis is a resinous bee product and

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consisted of different components which exhibits high antibacterial properties. Its mixture is variable and dependent on the season, location, and vegetation of collecting area (Eskandarinia, Rafienia, Navid, & Agheb, 2018). Its phenol compounds including flavonoids and phenolic acids have undeniable effects on the acceleration of the proliferation and remodeling phases of wound healing (Nani et al., 2018; Rosseto et al., 2017).

HA, a polysaccharide that is ubiquitous in the human body and has neutral effect on the immune system has been used for various clinical purposes such as drug delivery and tissue engineering (S.-J. Kim et al., 2017). It is a biopolysaccharide which consisted of repeating disaccharide units of D-glucuronic acid and *N*-acetyl glucosamine (Kogan, Soltes, Stern, & Mendichi, 2007; Menaa, Menaa, & Menaa, 2011). It is one of the main components of vertebrates' connective tissue, umbilical cord gel, synovial fluid, and especially rooster comb (Fakhari & Berkland, 2013). HA molecules form high percentages of the extracellular matrix and can absorb a significant volume of water. Therefore, it can play an essential role at the tissue hydration (Mero & Campisi, 2014). Also, its hygroscopic properties cause controlling of the hydration during periods of wound repair. HA derivatives improve the healing process in surgical wounds, burns, and chronic wounds by enhancing fibroblast proliferation. Also, HA loaded films induced secretion of skin-like materials by keratinocytes and fibroblasts (Chen et al., 2005; Li et al., 2018). HA has also been successfully used for cosmetic applications such as skin augmentation (Voigt & Driver, 2012).

In general, different materials have been utilized as the base of different wound dressings including starch and chitosan. Starch has more ability for sustaining wound moisture as a determinative factor for wound healing in comparison with chitosan (Y. Xu, Kim, Hanna, & Nag, 2005). However, it does not have antibacterial properties. The main aim of the present study is to improve starch-based film wound dressing by incorporation of propolis and HA. Propolis has significant anti-bacterial properties (Castaldo & Capasso, 2002). Grange et al reported that EEP can completely inhibit the growth and proliferation of many different Gram-positive and negative bacteria species (Grange & Davey, 1990). The mechanism of antimicrobial activity of propolis is complex and could be attributed to the synergistic activity between phenolic and other compounds (Krol, Scheller, Shani, Pietsz, & Czuba, 1993), mainly to the flavonoids pinocembrin, galangin, and pinobanksin (Castaldo & Capasso, 2002). In addition, it has many advantages over common antimicrobial agents like silver and copper, including high biocompatibility and natural origin (Martinotti & Ranzato, 2015; Torlak & Sert, 2013). Moreover, it exhibits significantly more antibacterial activity in comparison with other natural materials like green tea, cloves, and black tea extracts (Mohammad, 2013). In addition, propolis exhibits high antioxidants activity, tissue regeneration properties, immuno-stimulatory, and wound healing characterizations (Oršolić et al., 2012, 2013). Moreover, its plasticizing properties improves the flexibility and processability of the films (Bodini, Sobral, Favaro-Trindade, & Carvalho, 2013; Mathew & Abraham, 2008). HA was incorporated to the wound dressing structure for enhancing fibroblast proliferation and migration to accelerate the wound repair process. Incorporation of these materials to the CS films would be through physical interactions for better release in the wound environment. The CS/EEP films have exhibited appropriate characterizations according to *in vitro* evaluations in our previous study (Eskandarinia et al., 2018). Therefore, we decided to put one step beyond and incorporate HA for accelerating the wound healing process and investigate the new wound dressings at *in vivo* assessments. To the best of our knowledge, this is the first time to use the blended film dressings of CS/HA/EEP as a wound dressing with antibacterial and wound healing accelerating features. The blended film dressings were evaluated at physiochemical, mechanical and biological aspects *in vitro* and especially *in vivo* wound healing assay.

2. Material and methods

2.1. Materials

High amylose cornstarch (S4180) was obtained from Sigma Aldrich (CA, USA). Subsequently, the amylose/amylopectin ratio was measured using a commercial kit (Megazyme Co., Wicklow, Ireland) according to the manufacturer's recommendations. 71.7% amylose and 28.3% amylopectin was detected at the used cornstarch. The weight average molecular weight (M_w) and the number average molecular weight (M_n) of the cornstarch were determined by gel permeation chromatography (GPC, Waters 1515, USA) and were 4.3×10^5 Da and 2.5×10^5 Da, respectively. Also, the polydispersity index (M_w/M_n) of the used cornstarch was 1.72. Hyaluronic acid (HA) with a nominal molecular weight of 1.00×10^5 Da was purchased from Nanobio array corporation (Institute of Pasteur, Iran). GPC measurement in 0.1 M $NaNO_3$ water solution showed that HA has an M_n of 1.73×10^5 Da and M_w of 1.54×10^5 Da. The polydispersity index (M_w/M_n) of the utilized HA was 1.12. Ethanol 70% and glycerol anhydrous were purchased from Merck (Germany). Propolis was collected manually from the bee-hives in Isfahan, Iran. The chemicals such as Dulbecco's modified Eagle's medium (DMEM, 99.9% purity), fetal bovine serum (99.9% purity), 0.05% Trypsin EDTA, phosphate buffer saline (PBS, 99.9% purity) and 3-(4,5-dimethylthiazol- 2-yl-2,5-diphenyltetrazolium bromide) (MTT, 99.5% purity) were also purchased from Sigma Aldrich (USA). L929 cell line was purchased from Pasteur Institute of Tehran, Iran. Double distilled water was used as the solvent, throughout the experiment.

2.2. Preparation of ethanolic extract of Propolis (EEP)

Propolis was obtained from apiaries located in the suburbs of Isfahan, Iran. After harvesting, it was frozen for 24 h and then crushed in a blender. The sample was dissolved in 70% ethanol with a ratio of 1:10 (25 g of propolis in 250 ml of ethanol) and then was placed in an incubator at 37 °C for 14 days in a dark place according to previous studies (Ariamanesh et al., 2017; Shavisi, Khanjari, Basti, Misaghi, & Shahbazi, 2017). First, the suspension was filtered through Whatman No. 4 filter paper and it was frozen at −20 °C for 24 h. Then, the frozen suspension was slowly melted in room temperature and was filtered again with Whatman No. 4 filter paper for removing less soluble substances. Then, the suspension was placed in a rotary evaporator at 40 °C for removal of the solvent and the product was stored at 4 °C until further use.

2.3. Films formation

The films were produced by a casting technique. To prepare 2 wt% cornstarch solution, as the optimum concentration, 2 g of starch was solved in 100 mL of distilled water at 120 °C for 30 min. After solubilization, the plasticizing agent glycerol (20 g/100 g of starch) was added, and the solution was magnetically stirred (1 min) and kept for 15 min until the glycerol solubilization was completed (CS, pH = 6.81). After complete dissolution of starch in water, the filmogenic solution was cooled to 30 °C, and 1 wt% of hyaluronic acid was added to the mixture for 30 min (CS/HA, pH = 6.65). Then, 0.25 wt% (CS/HA/0.25%EEP, pH = 6.41), 0.5 wt% (CS/HA/0.5%EEP, pH = 6.32) and 1 wt% (CS/HA/1%EEP, pH = 6.02) ethanolic extract of propolis were added to the polymer/hyaluronic acid solution, and the mixture was again placed under a stirrer for 20 min. The solution was magnetically stirred. The filmogenic solution was distributed into acrylic plates (12×12 cm^2) and subsequently dried in a forced air oven at 37 °C, for 16 h. The produced gel was degassed with a vacuum pump (temperature, 40 °C, vacuum, 6 Pa) for 10 min, a cast in glass Petri dishes and dried at 50 °C for 24 h to form the film formation.

2.4. Attenuated total Reflectance/Fourier transform infrared spectroscopy (ATR/FTIR)

The chemical composition of the raw materials was evaluated by the infrared spectroscopy technique and to study the establishment of possible interactions between the compounds. The analysis was performed using an attenuated total reflectance (ATR) cell on the spectrophotometer FTIR-4200 type A (JASCO, USA), in a range of 500-4000 cm^{-1}, at a 4 cm^{-1} resolution with 64 scans.

2.5. Light transmission and opacity measurements

The visible-light transmittance curves through the film dressings (4 × 3 cm^2) were recorded in the range of 200–800 nm on an ultraviolet-visible (UV–vis) spectrophotometer (UV-2600, Shi-madzu, Kyoto, Japan). The opacity of the film dressings were calculated as follows:

$$\text{Opacity measurement} = \frac{A_{600}}{T}$$

Where A_{600} is the absorbance at 600 nm and T is the film dressing thickness (mm).

2.6. Gas Chromatography/Mass spectrometry

The ethanolic extract of propolis was analyzed using Gas Chromatography-Mass Spectrophotometry (7890 A, Agilent Technologies, Inc.). About 5 mg of the EEP was mixed with 50 µl of dry pyridine and 75 µl bis(trimethylsilyl) trifluoroacetamide, heated at 80 °C for 20 min and analyzed by GC–MS. Operative conditions were as follow: Silica fused capillary DB-5MS column, 30 m × 0.25 mm i.d., 0.25 µm film thickness (Agilent Technologies), helium as a carrier gas at a flow rate of 2 mL/min, oven temperature was programmed form 80–300 °C with linear increase of 10 °C/min and a final isotherm of 2 min, transfer line temperature was 280 °C, MS source temperature was 230 °C, MS quad temperature was 150 °C and ionization was performed at EI mode (70 eV). The spectrum was analyzed and compounds identified using the NIST05 data library.

2.7. Scanning Electron microscopy

Morphologies of the film dressings were characterized by utilizing scanning electron microscopy (SEM). The surface and cross-sectional morphologies were viewed using a Tescan Vega 3 LMH (Czech Republic). The film dressings were previously stored in a desiccator with P_2O_5, in order to eliminate the film dressing moisture. The film dressings (0.5 cm^2 approximately) were cryo-fractured by immersion in liquid nitrogen and mounted on cupper stubs. Samples were placed on a graphite ribbon that was fixed on aluminum sample holders and sputter-coated with gold in a modular high-vacuum coating Q150R ES (Quorum Technologies). After gold coating, the samples were observed using an accelerating voltage of 20 kV.

2.8. Mechanical properties

The mechanical properties were determined by tensile tests according to the ASTM method D882-10 (ASTM, 2010a) using an Instron Universal Testing Machine (Instron Engineering Corporation, Canton, MA, USA). Film dressing samples (120 mm × 25.4 mm) were fixed in a specific probe (tensile grips). The separation distance was kept at 100 mm, and the test speed was 50 mm/s.

2.9. Film thickness

Film thickness was determined using a digital micrometer (Model 102–301, Mitutoyo) at ten different positions of the film with 0.001 mm of accuracy.

2.10. Equilibrium swelling

The film dressing samples were cut into 2 × 2 cm^2 pieces and vacuum-dried at room temperature for 6 h. All film dressings were immersed in PBS (10 mM, 2.7 mM potassium chloride, 137 mM sodium chloride, and 1.76 mM potassium phosphate. pH 7.4) and kept at 37 °C. Swollen film pieces were lifted at various time points, till the equilibrium was achieved. The swollen films were removed and immediately weighed with a microbalance after utilizing a filter paper for absorbing the excess of PBS lying on the surfaces. The equilibrium swelling ratio (ESR) was calculated using the following equation:

$$\text{ESR} = \frac{(W_s - W_d)}{W_d}$$

The W_s and W_d are the weights of the films at the equilibrium swelling state and the dry state, respectively.

2.11. Hydrolytic and enzymatic degradation

The films resistance to hydrolytic and enzymatic degradation was evaluated by incubating the samples in two different aqueous mediums. Subsequently, their weight loss as a function of time was monitored. To evaluate the resistance against enzymatic degradation, the films were incubated in simulated wound fluid (SWF) (0.05 M, pH 7.4), supplemented with lysozyme from chicken egg white (0.25 mg/mL, 10,000 U/mL) at 37 °C for 72 h. The utilized enzymatic concentrations were similar to those found in human blood serum. Fresh lysozyme solution was added to each film for retaining its activity. The dry samples, accurately weighted (≈25 mg), were covered with 1 ml of the afore mentioned medium, freshly prepared, and left to rest in an oven at 37 °C. The enzyme medium was changed every 24 h. At predetermined intervals, samples were removed, washed with deionized water, and dried under vacuum at room temperature for 24 h. The samples were then weighted and the percentage of remaining weight was determined according to the mentioned equation: where Wo is the original weight and Wt is weight of the sample at the degradation time.

$$\text{Remaining Weight (\%)} = \frac{W_t}{W_o} \times 100\%$$

For assessing the films stability in an aqueous medium without enzyme (hydrolytic degradation), the samples were immersed in a PBS solution (pH 7.4), at 37 °C. The weight loss along time was determined following the same procedure reported above.

2.12. EEP release

The EEP release from the CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP film dressings were carried out after soaking in 5.0 mL PBS at 37 °C under the oscillation of 80 rpm. At the different time points (1, 2, 4, 6, 12, 24, and 48 h), 1.0 mL of the medium was extracted and the EEP concentration was analyzed by high-performance liquid chromatography (HPLC, Agilent 1100, U.S.). At the same time, the same volume of fresh PBS was added in the medium. The assays were performed in duplicate.

2.13. Antibacterial activity

In vitro antibacterial experiments were performed with the disc-diffusion method (C. Chen, Liu et al., 2018). The *Staphylococcus aureus* (ATCC 25,923), *Staphylococcus epidermidis* (ATCC 25,925), *Escherichia coli* (ATCC 25,922) and *Pseudomonas aeruginosa* (ATCC 27,853) were purchased from the Pasteur Institute of Tehran (Iran). The microbial suspensions of 1 × 10^8 cells/mL were inoculated in nutrient agar plates by spread plate method. The prepared samples (circular sheet, 6-mm diameter) were placed on the plates and incubated for 24 h at 37 °C. The plates with discs were monitored, and the clearance zones were

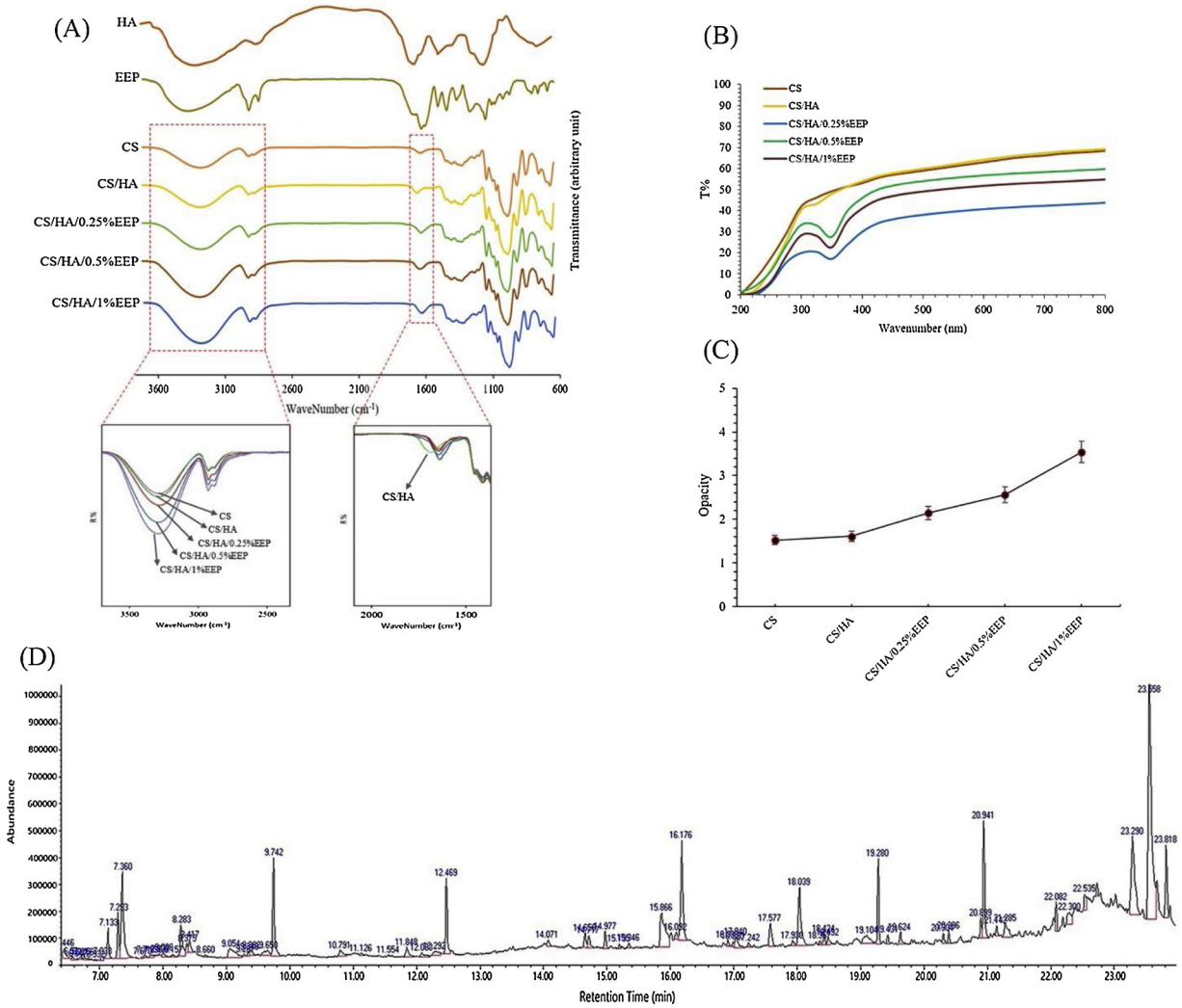

Fig. 1. (A) ATR-FTIR spectra of HA, EEP, CS, CS/HA, CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP film dressings. (B) UV and visible light barrier properties. (C) Opacity of the film dressings. (D) Chromatogram obtained from GC–MS with the ethanolic extracts of propolis.

measured.

2.14. Cell culture

The *in vitro* biocompatibility of the film dressings was evaluated with L929 murine fibroblast cells which were obtained from the Pasteur Institute of Tehran. The cells were cultured in RPMI supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin/streptomycin (Sigma, USA). The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. The samples were placed in home-made Teflon inserts that simultaneously fix the film dressings in place and define a circular seeding area with a diameter of 8 mm. The inserts were placed in 24-well cell culture plates. The cells were cultured at a density of 5×10^4 cells/ml per well in four different samples. Also, the L929 cells were seeded to the cell culture plates containing culture medium without any film dressing as the control samples. The culture medium was refreshed every two days for all groups. No adherent cells were removed by 3 times wash with PBS. After 4 h of incubation, 1 mL of fresh culture medium was added to each well.

2.15. MTT assay

The viability of cultured L929 fibroblast was monitored after 1, 4, and 7 days using MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide) colorimetric assay. To determine the toxicity of film dressings, cells were seeded into 96-well culture plates at 5×10^4 cells/well and they were incubated for 24 h. After 4 h incubation at 37 °C, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The dissolved solution was swirled homogeneously for about 10 min by the shaker. The optical density of the formazan solution was identified by a 96-well plate reader at 575 nm (Salehi et al., 2017).

2.16. In vivo wound healing experiments

The *in vivo* experiments were carried out using 32 female Wister rats (6–8 weeks old, 150–180 g). All animal experiments were performed in compliance with guidelines approved by the Animal Use and Care Administrative Advisory Committee of the Isfahan University of Medical Sciences. Before the wounds' creation, the rats were anesthetized with intraperitoneal injection of ketamine and xylazine mixture. The rats' dorsal region skin was depilated using an electric shaver and then, disinfected with 70% alcohol. The target areas were excised (12 mm in diameter) using a sterile surgical scissor and rats were randomly divided into four groups. Each rat was kept in separate cages with complete access to commercial animal lab food and water until they were sacrificed. Four groups (n = 8) including CS, CS/HA, CS/HA/0.5%EEP, and no-treatment (control) were prepared. The wounds of control group were just covered by sterilized cotton gauze. Each wound of the treatment group was covered with one film dressing (12 mm

diameter) which was precisely applied at the wound site and wrapped with sterilized cotton gauze. The wound healing was evaluated by continues wound diameters measurement by a digital caliper and capturing pictures on certain days (1st, 7th, and 14th days).

2.17. Statistical analysis

Statistical analysis was performed with SPSS (v17.0). All data were presented as a mean ± standard deviation indicated (mean ± SD), and statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Structural properties

ATR/FTIR spectrum of pure CS, HA, EEP, and the film dressings with different concentrations of ingredients (CS/HA, CS/HA/0.25% EEP, CS/HA/0.5%EEP, CS/HA/1%EEP) are shown in Fig. 1A. All film dressings had almost the same pattern of FTIR spectra. Although most of the peaks exhibited characteristics of cornstarch, different transmittance intensities were observed at certain peaks. The common bands of pure CS film dressing exhibited various peaks including obvious broadband spreading at 3150–3600 cm^{-1} due to O—H groups and 2893 and 2925 cm^{-1} which are due to C–H stretch. Also, the pure HA FTIR spectrum had a 3490 cm^{-1} peak which is corresponding to the stretching vibration of O—H groups. Two peaks at 1620 and 1410 cm^{-1} were observed which can be attributed to the asymmetrical C–O stretching and symmetrical C–O stretching of COO^-, respectively. The propolis extract spectrum showed typical hydrogen-bonded O—H stretch of phenolic compounds at 3336 cm^{-1} (phenolic hydroxyl group) and also absorptions at 1617, 1496, and 1450 cm^{-1} which are corresponding to the C=C stretches of the aromatic rings (Chang-Bravo, López-Córdoba, & Martino, 2014; Do Nascimento et al., 2016). EEP containing film dressings (CS/HA/0.25%EEP, CS/HA/0.5%EEP, CS/HA/1%EEP) exhibited stronger peak intensity at 2930 and 2870 cm^{-1} in comparison with pure CS film dressing. This fact is probably because of the stretching vibrations of the C–H bonds in CH_2 and CH_3 groups, respectively. As Fig. 1A illustrates, the shift at 1620 cm^{-1} indicates HA presence at the CS/HA structure. The comparison of the CS/HA/0.25% EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP with the pure CS exhibited that increasing of the EEP concentration and presence of the HA at the film dressing cause broader and higher peak at 3100-3600 cm^{-1} (Fig. 1A) (Chang-Bravo et al., 2014; El-Aassar, El Fawal, Kamoun, & Fouda, 2015). The increase of peaks intensity at the 3100-3600 cm^{-1} can demonstrate the incorporation of EEP, HA, and CS at the film dressings. According to FTIR analyzes, no evidence of chemical interactions or formation of new bonds was observed and these materials are interacting through physical interactions. Also, FTIR spectra of the film dressings confirmed the presence of free hydroxyl groups according to the peaks at 3100-3600 cm^{-1}, which are responsible for water holding capacity (Cyras, Manfredi, Ton-That, & Vázquez, 2008).

3.2. Light transmission and opacity measurements

Transmission of visible light at 200–800 nm wavelength was assessed for all film dressings Fig. 1B. Incorporation of EEP into the film dressings decreased the transmittance values in the UV and visible light region in comparison with the CS and CS/HA. This fact is probably due to the presence of phenolic compounds in EEP. CS/HA/1%EEP exhibited an excellent barrier effect for radiation in the UV/Visible region. Shojaee-Aliabadi et al. (2013) reported that the presence of the phenolic compounds in the ethanolic extract could absorb light at lower wavelengths (Shojaee-Aliabadi et al., 2013). The propolis structure is rich in unsaturated bonds which are responsible for the absorption of UV radiation (Bitencourt, Fávaro-Trindade, Sobral, & Carvalho, 2014). The absorption peak intensity was increased as the concentration of the EEP risen from 0.25 to 1%. Opacity is an established measure of film dressing transparency, and higher opacity means smaller transparency. As shown in Fig. 1C, the CS was relatively transparent (opacity = 1.52). The opacity of CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1% EEP film dressings drastically increased upon incorporation of the EEP up to 1 wt % in comparison with CS and CS/HA which reflects the influence of EEP incorporation into the film dressings. The main advantage of film dressing's transparency is to make it possible to observe and follow the wound healing process without removing the wound dressing.

3.3. GC–MS analysis

Propolis is a natural product with antimicrobial activities. Its bioactivities deeply depend on its chemical compositions. Therefore, in the current study, the chemical composition of the utilized propolis was analyzed using GC–MS. The profile of the EEP (appropriate MS data with high probability index > 90 were considered) are illustrated in Table 1S and Fig. 1D. The most common compounds in utilized EEP were fatty acids (palmitic acid, stearic acid, eicosanoic acid, and myristic acid), aromatic acid (p-coumaric acid and cinnamic acid), flavonoid (5,7-dihydroxy-2-phenyl-4H-1-benzopyran-4-one, pinocembrin, naringenin, vanillin, columbianetin), and phenolic acids (p-coumaric acid, 3-hydroxy-4-methoxycinnamic acid, caffeic acid). The results identified 5,7-dihydroxy-2-phenyl-4h-1-benzopyran-4-one (CAS: 480 − 40-0), 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl- (CAS: 18956 − 15-5), and myristic acid (CAS: 544 − 63-8) as the three major compounds of EEP and their levels were 14.78%, 7.78%, and 7.60%, respectively (Table 1S). There are a variety of antibacterial compounds in propolis which their quantities and quality are dependent on the extraction method (C. Chen, Liu et al., 2018). Caffeic acid as one of the main components of the utilized EEP was found to be an effective antibacterial agent with high activity against *S. aureus* (Yaghoubi, Ghorbani, Zad, & Satari, 2007; Zeighampour, Shams, & Naghavi, 2014). Therefore, the utilized EEP can exhibit considerable antibacterial activity due to the presence of phenolic compounds and flavanones as well-known antibacterial agents.

3.4. Microstructure

Fig. 2 shows SEM micrographs of the CS, CS/HA, CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP film dressings. The noticeable morphological difference was observed between the CS and CS/HA film dressings. The CS film dressings were found to have homogeneous and smooth structure. However, CS/HA film dressings exhibited rough surface. Similar microstructures have been observed at other film dressings with different compositions such as carrageenan/HA films (El-Aassar et al., 2015). Film dressings without EEP presented a more homogeneous surface (Fig. 2A and B) in comparison with EEP-containing film dressings (Fig. 2C-E). Increase of the EEP concentration increased films' surface roughness. According to the cross-sectional SEM micrographs (Fig. 2), incorporation of HA and EEP didn't affect the films porosity and to demonstrate this observation, dry density of the films was estimated (Table S2). No significant ($p > 0.05$) difference was observed between the films' dry densities. Therefore, incorporation of HA and EEP cause negligible impact on the films' porosity, but significant changes can be observed at the roughness of the films surface. Also, as it is apparent at the cross-sectional SEM micrographs, the pores of the films are not interconnected and limited. Therefore, the surface morphology of the films can be more attributed to roughness than porosity. Propolis is composed of different fatty acids (Table 1S) and its incorporation significantly affected the film microstructure leading to formation of heterogenous matrices which indicates the lack of lipid phase integration in the matrix at the used concentrations (Acosta, Jiménez, Cháfer, González-Martínez, & Chiralt, 2015). The rough surface of film dressings can enhance their efficacy for cellular attachment

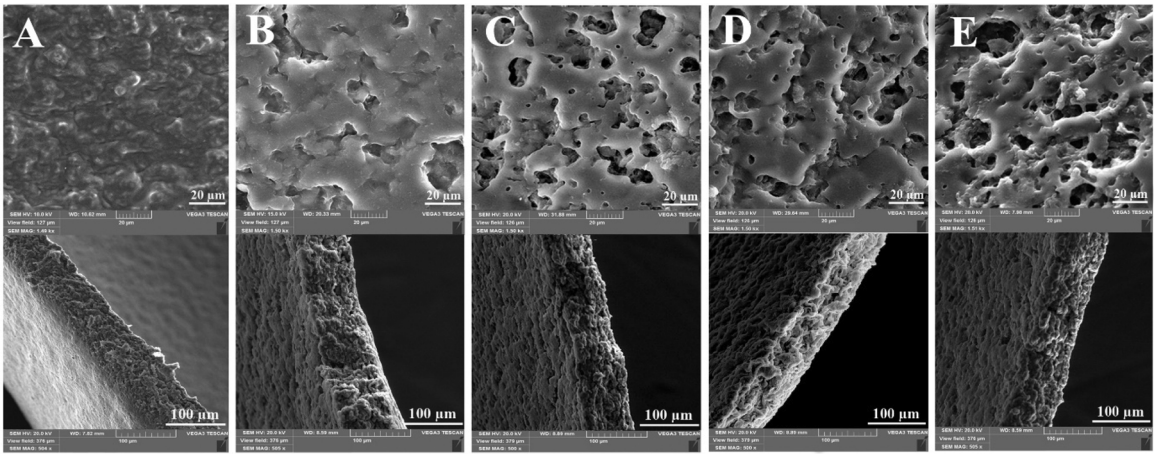

Fig. 2. SEM micrographs of the surface (top) and cross-section (down) of CS (A), CS/HA (B), CS/HA/0.25%EEP (C), CS/HA/0.5%EEP (D) and CS/HA/1%EEP (E) film dressings.

(Persson et al., 2014; C. Xu, Yang, Wang, & Ramakrishna, 2004).

3.5. Mechanical properties

The mechanical properties of film dressing can determine its handling efficacy. Therefore, assessment of these parameters is necessary for the biomedical applications. Table 1 illustrates the film dressing's tensile strength (TS) and elongation at break at different concentrations of ingredients. The thickness of the film dressings increased from 0.115 ± 0.017 μm to 0.128 ± 0.02 μm as the concentration of EEP increased from 0.25 to 1%. The thickness of the film dressings was completely dependent on the concentration of EEP. The elongation at break of CS/HA (41.71 ± 1.2%), CS/HA/0.25%EEP (42.91 ± 9.6%), CS/HA/0.5%EEP (68.24 ± 1.3%), and CS/HA/1%EEP (79.6 ± 7.5%) were about 1.12, 1.08, 1.78, and 2.31 times higher than the cornstarch film dressing (38.44 ± 4.7 MPa), respectively. The cornstarch film dressing without HA and EEP (CS) exhibited a TS value of 6.62 ± 1.2 MPa. According to previous studied, High amylose corn starch is known to produce strong films probably due to amylose crystallization (Mendes et al., 2016). These results are consistent with previous studies (Rubilar et al., 2013; Song, Shin, & Song, 2012). Although HA incorporation has no significant effect ($p > 0.05$) on the TS of the film dressing, an increase of EEP concentration can significantly ($p < 0.05$) decrease the TS value. This fact causes the formation of enlarging spaces between the polymeric chains and subsequent increase of the film dressings flexibility (de Araújo et al., 2015; El-Aassar et al., 2015). In general, the mechanical properties of the film dressings are closely associated with the distribution and density of inter and intramolecular interactions between the polymer chains in the film dressing matrix (Kanmani & Rhim, 2014). The EEP and CS/HA interactions are physical and there is no covalent bond formation. In addition, many studies have used EEP due to its plasticizing properties (de Araújo et al., 2015; Villalobos et al., 2017). These results suggest that EEP plays the plasticizing role which increases the mobility of the polymer chains and film dressing elongation and also, reduces the TS (Araújo et al., 2015). Although EEP incorporation to the film dressing decrease the TS, it is still high enough for application as wound dressing (Lee et al., 2016).

Table 1
. The mechanical properties of different film dressings.

Sample	Thickness (mm)	Elongation at break (%)	Tensile strength (MPa)
CS	0.115 ± 0.017[a]	38.44 ± 4.7[a]	6.62 ± 1.2[a]
CS/HA	0.116 ± 0.039[a]	41.71 ± 1.2[b]	6.34 ± 0.5[a]
CS/HA/0.25%EEP	0.115 ± 0.084[a]	42.91 ± 9.6[b]	6.08 ± 1.5[a]
CS/HA/0.5%EEP	0.121 ± 0.072[b]	68.24 ± 1.3[c]	4.47 ± 0.9[b]
CS/HA/1%EEP	0.128 ± 0.02[c]	79.6 ± 7.5[c]	3.39 ± 1.4[c]

[a−c] Means in the same column with the same letter are not significantly different ($p < 0.05$).

3.6. Equilibrium swelling

Evaluation of the swellability of wound dressings is a critical parameter for wound infection control (Aramwit, Muangman, Namviriyachote, & Srichana, 2010). The equilibrium swelling ratio of the film dressings was determined in PBS (Fig. 3A). For film dressings without EEP (CS and CS/HA), the equilibrium swelling reached its highest values within the first 90 min. However, CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP reached to this point within 240 min. The main aim of this test is to determine the samples swelling equilibrium. Different studies have used different time periods according to their materials' features and the test was continued till the equilibrium was achieved. In this study, although all samples were reached to their swelling equilibrium in 4 h, the test was continued for 8 h to make sure no change will be observed. The EEP incorporation (0.25–1%) to the CS/HA film dressing caused a significant ($p < 0.05$) decrease in the swelling values. However, CS and CS/HA did not exhibit any significant difference at equilibrium swelling values ($p > 0.05$). These results are consistent with previous studies (Araújo et al., 2015; Tan, Chu, Payne, & Marra, 2009). Presence of hydrophobic compounds, such as terpenes and fatty acids at EEP can decrease the film dressings swelling by placing between polymer chains (Araújo et al., 2015). On the other hand, CS and HA have an abundant number of hydrophilic groups such as hydroxyl, amino, and carboxyl groups which can cause film dressing hydration (El-Aassar et al., 2015).

3.7. Hydrolytic and enzymatic degradation

The main aim of the degradation assessment is to determine the required time for degradation of dressings. Therefore, hydrolytic and enzymatic degradation of films were studied in PBS and medium simulating wound milieu supplemented with lysozyme enzymes, respectively. CS/HA/EEP immersed in PBS lost 22–31% of their weights within the first week and 33–52% of total mass remained until the 14th day (Fig. 3B). At day 28, the weight remaining percentage for all the film dressings was ranged from 0% to 12%. The degradation rate had reverse relation with EEP concentration in CS/HA/EEP films due to the hydrophobic properties (Coneac et al., 2008; Shi et al., 2015) and it was significantly more than CS and CS/HA ($p < 0.05$). The CS/HA/EEP samples which were immersed in PBS (pH 7.4) exhibited slower

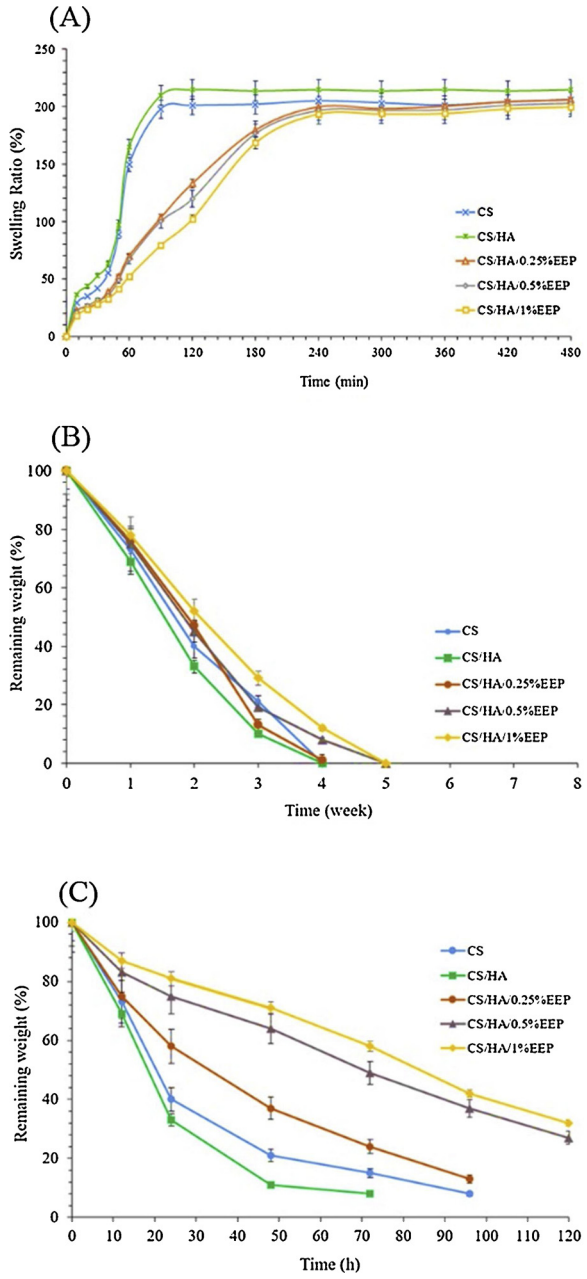

Fig. 3. (A) Effect of HA and EEP on equilibrium swelling ratio of film dressings with respect to time. Data are expressed as mean ± SD (n = 3). (B) Remaining weight of the film dressings in PBS. (C) Remaining weight of enzymatic degradation in SWF containing lysozyme. Data are expressed as mean ± SD (n = 5).

degradation (Fig. 3B) in comparison with the enzymatic medium immersed films (Fig. 3C). The film dressings immersed in SWF containing lysozyme enzymes shown 26–76% weight loss in the 24 h. The weight losses were found 87%, 73% and 68% for CS/HA containing 0.25%, 0.5% and 1% w/v of EEP, respectively. The rate of enzymatic hydrolysis decreased with increasing of the EEP concentration. Appropriate degradation of wound dressing (Bhargava, Patterson, Inman, MacNeil, & Chapple, 2008; Eke, Mangir, Hasirci, MacNeil, & Hasirci, 2017) can cause dissolving of the ingredients to the wound bed which can cause cell proliferation and construction of new skin.

3.8. Release of EEP

For the EEP release assessment, the CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP were immersed in PBS, and the EEP release was characterized by HPLC. All samples exhibited similar EEP release trends (Fig. 4). The EEP was released with high acceleration within 4 h, and subsequently, the release rates entered a steady state at next hours. After 48 h, the concentrations of EEP that were released from the CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP reached at 60, 69 and 74%, respectively. Therefore, the EEP as the main antibacterial agent of the films is slowly released in the wound environment. The slow and sustained release of the EEP can maintain the antimicrobial condition in the wound along the healing process.

3.9. Antibacterial activity

The main concern during the wound healing process is microbial contamination. Therefore, antimicrobial activity is the prerequisite condition for any wound dressing material. Antimicrobial activity of CS/HA/EEP film dressings against *Escherichia coli*, *Staphylococcal aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were determined by the disk inhibition zone assay, and the results are shown in Fig. 5 and Table 2. None of the film dressings could exhibit any zone of inhibition for *Pseudomonas aeruginosa* strains. Also, no inhibition zone formed on the solid plates of the CS and CS/HA which suggests that CS and HA have no antibacterial activity. However, CS/HA film dressings containing EEP exhibited antimicrobial activity on the contact surface underneath film dressings. Although clear zones surrounding CS/HA/0.25%EEP was observed, the antibacterial effects were far from that desired. The CS/HA/0.5%EEP and CS/HA/1%EEP exhibited the most antibacterial effect between the film dressings. The best activities were against *Escherichia coli* (4.33 ± 0.27 mm), *Staphylococcal aureus* (4.68 ± 0.12 mm) and *Staphylococcus epidermidis* (2.92 ± 0.26 mm). *S. epidermidis* has exhibited smaller halo in comparison with *E. coli* and *S. aureus*, which can be related to the surrounding polysaccharide layer, slime layer, which decrease the entrance of antibiotics and antibacterial agents to the bacterial cells (Patrick, Plaunt, Hetherington, & May, 1992). In addition, *P. aeruginosa* did not exhibit any sensitivity to the EEP containing films which can be attributed to the mucoid layer of alginate exopolysaccharide (Costerton, Stewart, & Greenberg, 1999; Leid et al., 2005). This substance is excreted by the bacteria and can cause resistance to antibacterial agents. The previous studies have attributed the antimicrobial effect of EEP film wound dressings to flavonoids and aromatic acids and their esters (Siripatrawan, Vitchayakitti, & Sanguandeekul, 2013) which were identified by the GC/MS in our utilized propolis.

3.10. Cytotoxicity

The cytotoxicity of the film dressings was evaluated by MTT assay (Fig. 6). MTT assay is an important technique to evaluate the biocompatibility of different biomaterials. L929 fibroblast cells were cultured on different film dressings for 1, 4, and 7 days (Fig. 6A). None of these dressings caused cytotoxicity which is a crucial property for their future application in wound healing (Lönnroth, 2005). Incorporation of HA to CS film dressings caused significant ($p < 0.05$) increase in the cells' proliferation after 7 days. Also, the most pro-proliferative effect on the L929 fibroblast cells was observed at this film dressing (CS/HA) which can be mostly attributed to HA (Sahiner, Sagbas, Sahiner, & Ayyala, 2017). However, incorporation of EEP (CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP) decreased cells proliferation in comparison with CS/HA film dressings. After 7 days of incubation, 1 wt % EEP containing film dressing could significantly ($p < 0.05$) inhibit L929 cells proliferation in comparison with CS/HA wound dressing. This result may be attributed to the release of unfavorable forming materials of EEP from the film dressings of CS/HA/EEP (J. I. Kim, Pant, Sim, Lee, & Kim, 2014). Therefore, CS/HA/0.5%EEP is a suitable amount to fabricate a biocompatible film dressing.

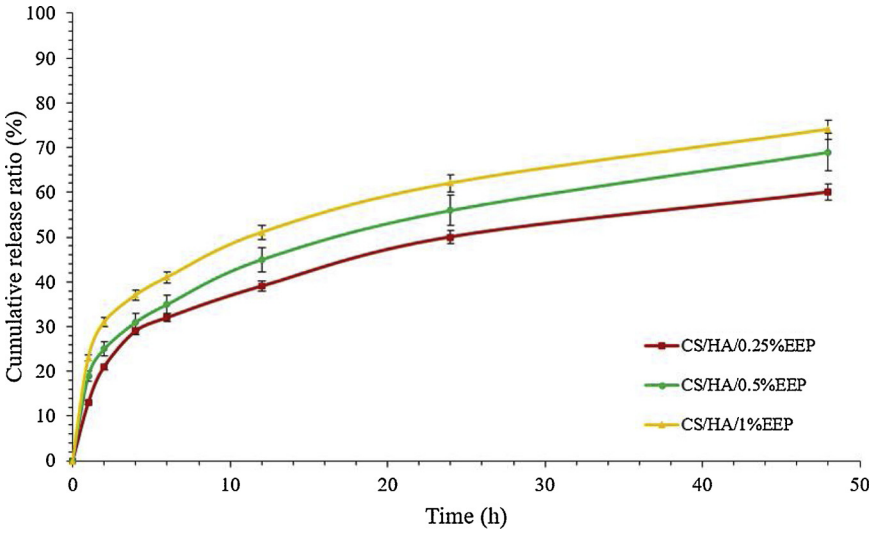

Fig. 4. Propolis release profiles of the film dressings.

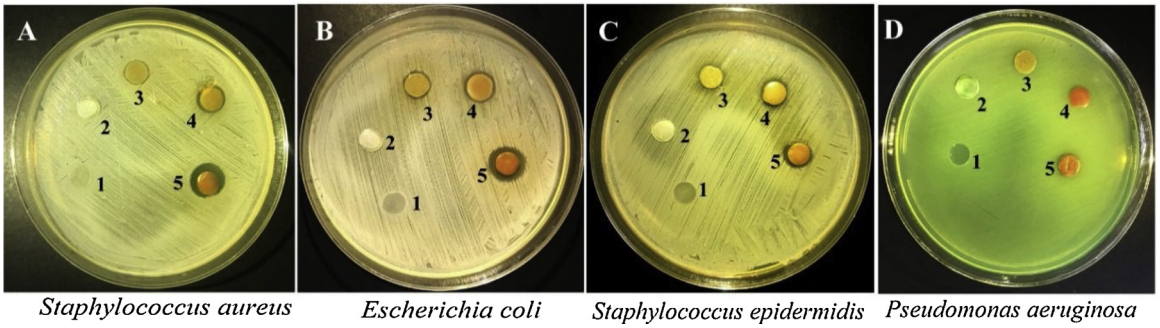

Fig. 5. The inhibition zones of different film dressing against *S. aureus*, *E. coli*, *S. epidermidis* and *P. aeruginosa* after a 24 h incubation are shown in A, B, C, and D respectively. (1) CS, (2) CS/HA, (3) CS/HA/0.25%EEP, (4) CS/HA/0.5%EEP and (5) CS/HA/1%EEP.

Table 2
Antimicrobial activity of CS film dressings with HA and EEP determined by inhibition zone method.

Microorganism	Inhibition zone (mm)				
	CS	CS/HA	CS/HA/ 0.25%EEP	CS/HA/0.5%EEP	CS/HA/1%EEP
S. aureus	0	0	0.93 ± 0.25	2.08 ± 0.14	4.68 ± 0.12
E. coli	0	0	1.21 ± 0.39	2.64 ± 0.18	4.33 ± 0.27
S. epidermidis	0	0	0	1.02 ± 0.15	2.92 ± 0.26
P. aeruginosa	0	0	0	0	0

3.11. Wound healing effect

The promoting wound healing effect of the CS/HA/EEP films was evaluated in Wistar rats' model experiment. The compounds should have a uniform distribution all over the film to cause equal effect on all parts of the wound bed. All the films were produced from completely clear solutions which exhibits appropriate desolvation of the compounds in the solvent without any aggregation. This fact can demonstrate uniform distribution of the compounds all over the film structure which cause their high transparency. The wounds were monitored for 14 days, and their images were captured at 1st, 7th and 14th day post operation (Fig. 7). All the rats survived throughout the postoperative period until sacrifice. All experimental groups began to show reductions in open wound area from day 7. The healing process for the wounds which were treated by film dressings were without any apparent complications, and no evidence of necrosis was observed. The observations demonstrated that the film dressings' degradation behavior at *in vivo* environment was consistent with the obtained results from the biodegradability test. Fig. 7A illustrates the macroscopic photographs of wounds treated with films dressings and gauze as the control. On day 14, the wounds treated with the films had approximately healed, whereas, this fact was not observed in control group. The CS wound dressing was able to adhere uniformly to the wound surface without the accumulation of wound exudates. Among the experimental groups, those treated with CS film dressing exhibited faster wound closure in comparison with the control (Fig. 7B). This fact can be attributed to high hydrophilic capacity of the CS films which maintains moist environment at the wound site (Archana, Singh, Dutta, & Dutta, 2013; Arockianathan et al., 2012). At each time points, wounds which were covered with CS/HA/EEP film dressing exhibited a more accelerated rate of wound closure than control. The film dressing absorbs the wound exudates, thus, reduces the risk of dehydration (H. Chen, Lan et al., 2018; Shi et al., 2015). Our study confirms that film dressings without any functional additives could act as a wound coverage material and promote wound healing. During the wound healing process, cornstarch gradually depolymerizes and releases propolis which initiates fibroblast proliferation and helps in order to collagen deposition and stimulates an increased level of natural hyaluronic acid synthesis at the wound site (Jayakumar, Prabaharan, Kumar, Nair, & Tamura, 2011).

4. Conclusions

In this study, hyaluronic acid and propolis incorporated cornstarch-based film dressing was synthesized and investigated. The CS/HA/0.5%

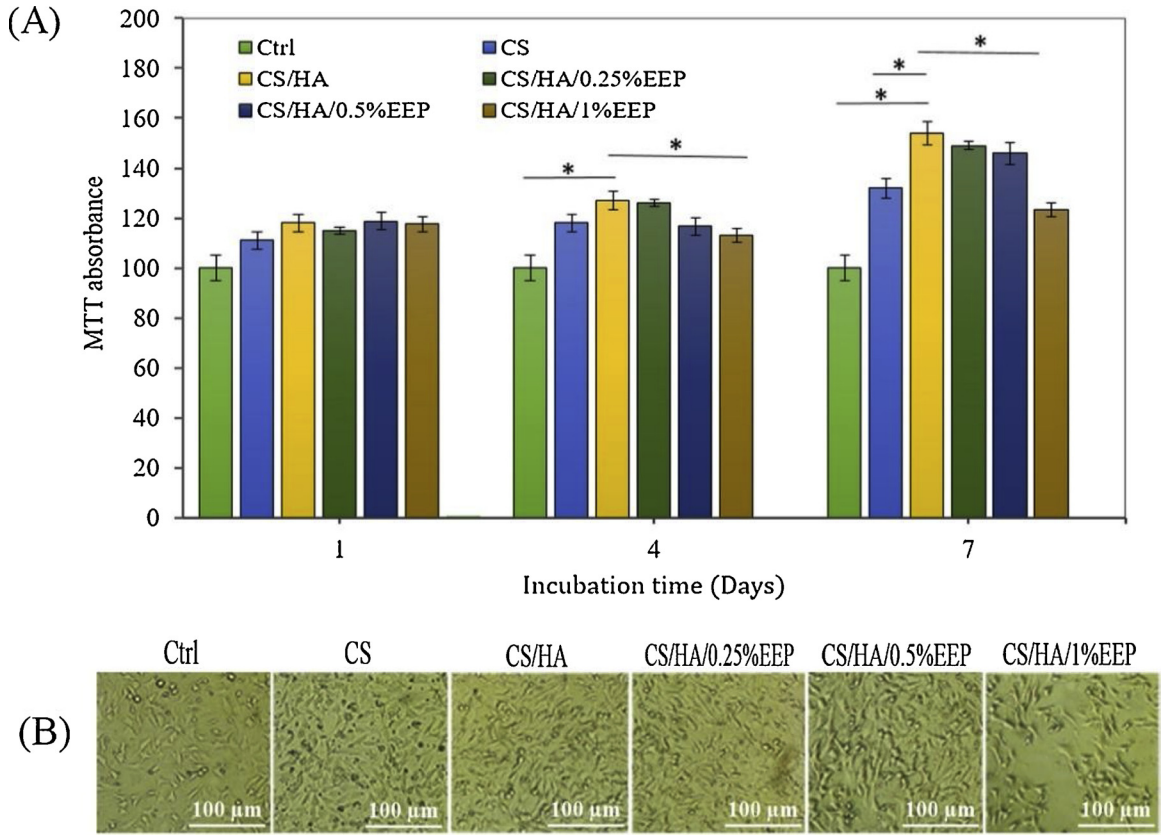

Fig. 6. (A) The fibroblast cell viability assay in the presence of cell culture medium (Ctrl), CS, CS/HA, CS/HA/0.25%EEP, CS/HA/0.5%EEP, and CS/HA/1%EEP film dressings after 1, 4, and 7 days incubation (* $P < 0.05$). (B) Optical microscopy images in the same magnifications of L929 cells treated with basic cell culture medium (control) and different film dressings for 24 h. The control is the L929 cells which were seeded to the culture plates containing culture medium without any film dressings.

EEP showed less swelling and degradation ratio in comparison with the CS and CS/HA dressings. This film dressing exhibited higher antibacterial activity against *S. aureus, E.coli,* and *S. epidermidis* in comparsion with the CS and CS/HA dressings. GC/MS analyzes demonstrated high antimicrobial agents' content of the utilized propolis like flavonoids which can explain the significant antimicrobial activity of the film dressing. In addition, CS/HA/0.5%EEP film dressing exhibited no cytotocixity for the normal murine fibroblasts. Moreover, the CS/HA/0.5%EEP film dressing caused considerable acceleration at the Wistar rats skin excisions healing. Therefore, hyaluronic acid and propolis incorporated cornstarch-based wound dressing has the potential to be used as an effective wound dressing material for skin repair.

Conflict of interests

There is no conflict of interests to declare.

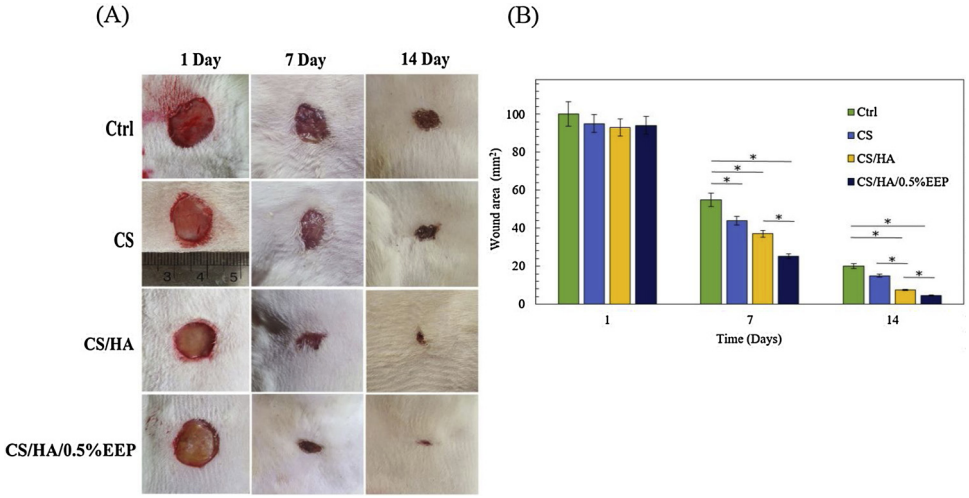

Fig. 7. (A) Wounds' photographs on days 1, 7, and 14 after full-thickness skin excision. (B) Wound closure progression (1–14 days). The data are presented as mean ± SD (n = 8). * shows the significant difference between groups at $p < 0.05$.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2019.03.091.

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