ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Wound Healing Potency Of Bacterial Cellulose Membranes Loaded With Different Antibiotics.

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ABSTRACT

Bacterial cellulose (BC) is a natural polymer used in wound healing. It was obtained from *Komagataeibacter hansenii.* Gentamicin, ampicillin and chloramphenicol at different concentrations were loaded into BC membranes. BC membranes loaded with different antibiotics were chemically characterized and investigated for their antibacterial and wound healing properties. The results elucidated high water holding capacity of BC membrane. In addition, the antibiotics loading capacities of BC membranes at different concentrations were higher in gentamicin followed by ampicillin then chloramphenicol. *In vitro* antibacterial effects were carried out against some Gram-negative and Gram-positive bacterial strains. BC-Chloramphenicol membrane was active against all used bacterial models. BC-Ampicillin membrane showed activity against all models of bacteria except *Staphylococcus aureus,* while BC- gentamicin membrane was active against *Pseudomonas aeruginosa* and *Bacillus subtilis.* BC and BC membranes loaded with different antibiotics were investigated for their wound healing potency in wounded rat model for 14 days. These membranes reduced the inflammation through regulating the expression of intercellular adhesion molecule 1, tumor necrosis factor -α and interleukin-6.This was followed by enhancing wound tissue collagen I, hydroxyproline, total protein, fibronectin and vascular endothelial growth factor with a reduction in tissue myeloperoxidase so they can be used as perfect dressing materials.
Keywords: Bacterial cellulose; Antibacterial; Wound healing.

https://doi.org/10.33887/rjpbcs/2019.10.3.45

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INTRODUCTION

Loss of the skin as one of the greatest body barriers triggers a mechanism for wound healing to recover the functions of the skin and to eliminate scar tissue formations [1]. Efforts have been made to regenerate human skin and to deliver therapeutic compounds during wound healing process. One of the materials which act as skin substitute, is bacterial cellulose [2]. Bacterial cellulose (BC) is a natural, renewable raw material polymer which is composed of glucose units linked together by β-(1→4)-glycosidic linkages with the formula $[(C_6H_{10}O_5)n]$ [3]. BC was produced as extra-cellular product by Acetobacteraceae, particularly *Komagataeibacter* genus, formerly known *Gluconacetobacter*, viz.: *Komagataeibacter xylinus* and *Komagataeibacter hansenii*, *Komagataeibacter swingsii*, *Komagataeibacter rhaeticus* and *Komagataeibacter medellinensis* [4, 5]. BC can be used as a substitute of plant cellulose (PC) because PC contains hemicellulose, lignin and pectin that hinders its employment in medical applications [6]. BC has a noticeable role in wound healing process due to its purity, high porosity with large surface area and its hydrophilic nature that maintains moisture in wound area [3]. Moreover, it is nontoxic, non-allergenic and biocompatible possessing high mechanical strength. BC is also considered as an ideal drug carrier matrix based on its unique nano-fibers network structure with fiber diameter ranging between 50–120 nm [7]. Besides, the natural performance of BC makes it an excellent wound healing material as it has the capability for cleaning wound exudates with no damage to the newly formed epithelial lining skin [8, 9].Wound repair involves the timed and balanced activity of inflammation and vascularization of connective tissue and epithelial cells [1]. In the wound microenvironment, vascular epidermal growth factor (VEGF) induces wound repair by aiding in vascular permeability, thus permitting inflammatory cells to reach the wounded site and enhances the proliferation and migration of the already present endothelial cells. BC membranes have been found to enhance VEGF production due to its intensified angiogenesis and accelerated healing performance [10]. Hydroxyproline content is used as a marker of the quantity of collagen in tissue and is enhanced by applying BC membranes in wound areas [11]. Fibronectin (FN) is believed to play a crucial role in directing regenerative wound healing cascade. FN has been shown to stimulate angiogenesis, reduce the inflammatory response, and increase the rate of wound closure [12]. Additionally, in wounds, reactive oxygen species (ROS) are generated due to the activation of neutrophils that also produce enzymes such as myeloperoxidase. Increasing wound myeloperoxidase activity causes damage to the extra cellular matrix proteins [13]. Even though, BC membrane possesses physical and mechanical properties that accelerates healing and reduces scarring by regeneration rather than repair [1] yet, it lacks antibacterial and antimicrobial effects resulting in its failure to provide a barrier against wound infection. This limits its application in wound healing area [14]. In addition, wounds are vulnerable to wound infections and inflammation due to high levels of inflammatory cytokines such as tumor necrosis factor -α (TNF-α) and interleukin-6 (IL6). The elevated levels of these cytokines delay healing causing less migration and proliferation of fibroblasts and keratinocytes, reduced accumulation of collagen and deferred re-epithelialization [15]. In the present study, three antibiotics have been impregnated into BC membranes and investigated for their wound healing potency in wounded rat model. Gentamicin (GM) which is an aminoglycoside molecule that exhibits bactericidal activity against a broad spectrum of micro-organisms [16]. Gentamicin was issued clinically in the treatment of infected skin cysts, ulcers, burns, infected insect bites and stings, infected lacerations, and wounds. The bacterial resistance to gentamicin is lower than other aminoglycosides [17].Chloramphenicol (CH) is another antibiotic that has a broad spectrum of activity against Gram-positive and Gram-negative bacteria. Chloramphenicol is suggested in treatment of sutured wounds infection [18]. In addition, Ampicillin (AM), which has broad antibacterial activity against pathogenic bacteria where different membranes loaded with Ampicillin, demonstrated high haemocompatibility and broad antibacterial activity suggesting their potential application in wound care [19]. Therefore, the present work aimed to produce BC membranes from *Komagataeibacter hansenii*. Then to load GM, AM and CH into BC membranes at different concentrations. To characterize BC membranes loaded with different antibiotics chemically then investigate their antibacterial activities against different bacterial strains. Moreover, to biologically evaluate the possible anti-inflammatory and wound healing potency of BC membranes alone or impregnated with GM, AM and CH in wounded rat model.

MATERIALS AND METHODS

Microorganisms and antibiotics

Komagataeibacter hansenii AS.5 (ac: MH109871) was used for production of BC under static condition. This strain was previously isolated from rotten apple and identified according to morphological, biochemical and genotyping characterization and maintained on HS-agar slants at 4°C. Gentamicin (GM) sulfate, Chloramphenicol

ISSN: 0975-8585

(CH) and Ampicillin (AM) sodium salts were used for antibacterial assay against to *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus* that were supplemented from Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Application, Alexandria, and maintained on LB solid agar medium at 4 °C.

Pre inoclume preparation

Hestrin & Schramm (HS) medium, that contained g/l: (D-glucose 20, peptone 5, yeast extract 5, sodium phosphate dibasic 2.7, citric acid 1.15 and ethanol 5 ml/l) were incubated at 30 ◦C for 150 rpm for 2 days. This was used for activation of *K.hansenii* AS.5 for production of BC [20].

Production and purification of BC membrane

Production of BC membrane was performed by growing *K.hansenii* AS.5 in HS medium at 30°C, pH 6 for 7 days with the inoclume size 10% from pre inoclume preparation, under static condition in 250 ml Erlenmeyer flasks containing 50 ml media. BC formation was monitored by the appearance of a white pellicle on the surface of the culture broth (a layer of BC in the interface of liquid/air). After incubation time BC pellicles were harvested and washed three times with distilled water to remove residues of media composition. Subsequently, BC pellicles were heated in 0.5% NaOH solution at 90°C for 30 min to remove the attached cells and other impurities immobilized on BC membranes and then washed 3-4 times with distilled water until reaching neutral pH. After that, the purified BC was dried at 70°C over night until constant weight is achieved [21]. Then the dry weight of BC was determined. Finally, the BC membrane was sterilized by autoclaving at 121 °C for 20 min [22] and stored at 4 ◦C in the refrigerator for further study.

Water holding capacity of BC membrane (WHC)

WHC was determined by freezing BC membrane (1 cm^2) at -80 for 24 h, after that the frozen BC membrane was immersed in 50 ml phosphate buffer solution (PBS) overnight to swell up completely. Then, BC membrane was removed from PBS and wiped slightly once with tissue to remove the excess of PBS from the surface of BC membrane and then the hydrated BC membrane was weighted to determine the water uptake and then dried at 80 °C until constant dried BC membrane weight was achieved in order to remove the water completely. The WHCs of different samples were calculated using the following formula:

$$WHC (\%) = (W_h - W_d) / W_d \times 100$$

Where W_h hydrate weight of BC membrane and W_d dry weight of BC membrane. The results were performed as the average of four samples [23, 24]

Impregnation of antibiotics into BC membrane

Antibiotic concentrations were loaded into BC membranes by swelling diffusion method [24]. The sterilized and dried discs of BC membranes (1 cm^2) for antibacterial assays and wound healing processes were immersed in different antibiotics (GM, AM and CH) at different concentrations (0.025, 0.050, 0.075 and 0.100 mg/ml). They were incubated in a shaking incubator at 37 °C and 100 rpm for 24 hours in order to assure complete absorption of BC with the antibiotics. GM solution was covered with aluminum foil and CH concentrations were dissolved in absolute ethanol. After that, the disc of BC films were removed from different antibiotic solutions and then immersed briefly in distilled water for 10 seconds and wiped slightly once with sterilized tissue to remove non-absorbed antibiotics from the surface of BC. Finally, the disc of BC-LA membranes were placed in a Petri dish and dried at 30 ◦C and kept at 4 °C in the refrigerator until they were used [24].

Antibiotics loading capacity of BC membrane

In this experiment, three pieces of frozen dried BC membrane (1 cm^2) were weighted up and immersed in 10 mL of different antibiotic concentration solutions at 37 °C , 100 rpm for 24 hours. Then BC membranes were taken from the solutions, immersed in 1 mL distal water for 10 seconds and treated with filter paper for the removal of extra free water on the BC membrane. Subsequently, the dried BC discs impregnated with antibiotics at different concentrations were weighed directly. The amount of the absorbed antibiotics in BC

membranes were determined indirectly by comparing the difference between the weight of BC dry before impregnation and the weight of impregnated BC in different antibiotic solutions. The antibiotic loading capacities of BC membranes (mg/cm^2) were calculated as the amount of the absorbed antibiotic concentrations per square centimeter of BC dry membrane [25].

Assay of antibacterial activity

The antibacterial activities of the GM, AM and CH containing dry BC discs (1 cm^2) were investigated by disc diffusion technique [26] against *E. coli* and *P. aeruginosa* as a Gram-negative model bacteria and *S. aureus and B. subtilis* as Gram-positive model bacteria. Disc diffusion approach was performed in a Petri dish containing Luria–Bertani (LB) medium which was composed of (g/l): peptone 10, yeast extract 5, sodium chloride 5 and agar 20. The dry BC discs impregnated with antibiotics at different concentrations were placed on *E. coli*, *P. aeruginosa, S. aureus* and *B. subtilis* cultured spread (optical density at 660 nm reached 0.2.) on agar plates (one disc per plate). BC membranes without impregnated antibiotics were used as controls. The agar plates were then incubated at 37 °C for 48 h and the antibacterial activity of BC films were photographed and determined by measuring the diameter of inhibition zone and compared with their controls. Triplicate experiments were done for each membrane [27, 28].

Characterization of BC-LA membranes

The morphological structure of BC membranes loaded by antibiotics at minimum concentration was performed by Scanning Electron Microscope (SEM) (JEOL JSM 6360 LA, Japan). BC-LA membranes were coated with a thin layer of gold nanoparticles in preparation for SEM imaging. SEM experiment was conducted at an accelerated voltage of 15 kV at a magnification of 5000 or 4000 X. The chemical interactions between BC membranes and antibiotics as well as functional groups and chemical bonds of dried BC-LA membranes were identified by Fourier transform infrared (FTIR) spectroscopy (Shimadzu FTIR-84 00 S, Japan), the scan range was from 4000 to 500 cm^{-1}, using a resolution of 1 cm^{-1}. The thermal decomposition behaviors of pure BC and BC-LA membranes were determined by thermogravimetric analysis (TGA) (Shimadzu TGA-50, Japan). Samples were conducting in a N_2 purge (40 ml/min) in a heated temperature from 25 °C to 800 °C at a heating rate of 10 °C min^{-1}.

Experimental Biological studies

Housing of experimental animals

Adult male Sprague Dawley rats weighing (100–110 grams) were bred and maintained under conventional conditions at the experimental animal research unit of National Research Center, Egypt. Animals were kept under a 12:12 light/dark cycle with a temperature of 23–25 ℃. Fresh tap water was available all time. Rats were acclimatized to laboratory conditions for a week before the beginning of the study. All animal procedures were performed in accordance with the guidelines for the care and use of experimental animals established by the National Institutes of Health (NIH) protocol (NIH Publications No. 8023, revised 1978) approved by the National Research Center.

Excisional and wound preparation

Wounding and wound healing studies were conducted on a total number of 30 male Sprague Dawley rats. Rats were anesthetized by giving an intramuscular injection of a mixture of 90 mg/kg of ketamine and 10 mg/kg of xylazine. The dorsal surfaces of rats' skin were shaved and sterilized with 5% povidone/iodine solution. 1cm ×1cm=10mm area of wound was induced to the dorsal area at the back of each rat by excising the skin and the underlying panniculus carnosus. The induced wounds in the first experimental group were left uncovered but the induced wounds in different experimental groups were covered with BC membranes (1cm ×1cm) either alone or impregnated with 0.025mg/ml of GM, AM and CH antibiotics using standard gauze and 3 M adhesive tape. As a control, similar amounts of skin were collected from the backs of non-wounded normal rats.

Animal grouping

Rats were divided into 6 experimental groups: Normal control group (NC), wounded group (W) where the wounds of this group were left uncovered without any membranes. (W/BC) group where their wounds were covered with BC membranes. Wounds of the fourth group (W/BC-GE) were covered with BC membranes loaded with GE. Wounds of the fifth group were covered with BC membranes loaded with AM (W/BC-AM). Wounds of the sixth animal group (W/BC-CH) were covered with BC membranes loaded with CH.

Macroscopic examinations of wound areas and measurements of wound area closure

Wound sites of all animals were photographed using a digital camera and the wound diameter was measured at 3, 7 and 14 days using vernier caliper. The percentages of wound area closure was estimated at same time intervals according to the following equation [29].

$$\text{Percentages of wound area closure} = 100\times (A1-A)/ A1.$$

A1 is the initial wound area calculated on day 0 and A is the wound area on 3, 7 and 14 days.

Inflammatory parameters

At 7 and 14 of experimental study period, five rats from each group were food deprived; over-night fasted, anesthetized with diethyl ether and dissected. Another five non-wounded rats for the NC group were similarly dissected. Blood samples were withdrawn by heart acupuncture from each group in clean dry test tubes. Then, blood samples were centrifuged at (14 000g) for 10 minutes. Inflammatory parameters were assessed according to the manufacturer's instructions as follows: Rat Intercellular Adhesion Molecule 1 (ICAM-1) concentration in plasma was assayed by intercellular adhesion molecule 1 ELISA kit catalog number.CSB-E04576r. CUSABIO, College Park MD. Rat tumor necrosis factor-alpha (TNF-α) concentration in plasma was assayed by rat TNF-α ELISA kit code number. 27194. Immuno-Biological Laboratories Co., Ltd. Japan. Rat interleukin-6 (IL-6) concentration in plasma was assayed by rat IL-6 ELISA kit code number.27768.Immuno-Biological Laboratories Co. ,Ltd. Japan.

Measurements of wound healing parameters

At the end of the study period 14 days, all the membranes were removed and areas that included the wound, the complete epithelial and dermal compartments of the wound margins, the granulation tissue and parts of the adjacent muscle and subcutaneous fat tissue were excised from each individual wound. As controls, similar amounts of skin were collected from the backs of non-wounded normal mice to be compared with all groups. These wounded areas from all experimental groups were harvested and frozen in liquid nitrogen. The specimens were homogenized in PBS and centrifuged at 5000×g for 10 minutes. The supernatant was removed and assayed according to the manufacturer's instructions as follows: Rat Collagen I concentration in wound tissue homogenate was determined using rat Collagen I ELISA kit catalog number.CSB-E08084r.CUSABIO,College Park, MD. Rat hydroxyproline concentration in wound tissue homogenate was assayed using rat hydroxyproline ELISA kit catalog number.CSB-E08838r. CUSABIO, College Park, MD. Rat Fibronectin (FN) concentration in wound tissue homogenate was determined using rat fibronectin ELISA kit catalog number.CSB-E04553r. CUSABIO, College Park, MD. Rat vascular endothelial growth factor (VEGF) concentration in wound tissue homogenate was determined using vascular endothelial growth factor ELISA kit code number.27101. Immuno-Biological laboratories Co., Ltd. Japan. Rat myeloperoxidase (MPO) concentration in wound tissue homogenate was determined using rat myeloperoxidase ELISA kit catalog number.CSB-E08722r. CUSABIO, College Park, MD. The protein content in wound tissue homogenate was estimated according to the method of Lowery et al. [30] using reagent kits obtained from Spectrum Diagnostic, Hannover, Germany.

ISSN: 0975-8585

Statistical analysis

Statistical analysis was performed through the statistical package for social science (SPSS) version 19 using Analysis of variance (ANOVA), followed by Tukey's post-test. Data were expressed as mean ± standard error of means (SEM).

^{a} $p < 0.05$ for significant results compared to NC group, ^{b} $p < 0.05$ for significant results compared to W group, ^{c} $p < 0.05$ for significant results compared to W/BC group

RESULTS AND DISCUSSION

Water holding capacity (WHC)

WHC is the most important parameter involved in the biomedical applications of BC membrane as a dressing material. The moisture content properties of a dressing material enhances the wound healing process and protects it from contamination [23]. Additionally, BC-LA as antibacterial membrane is capable of high water absorption. This is an important feature to promote wound healing [31]. As well as, facilitate the penetration of active substances such as antibiotics into the wound area without damage to the newly formed skin [32]. BC membranes prepared in this study could hold water at 231.8 mg/cm^2 which represents at least 105.3 folds its own weight of dry BC membrane after 24 h. Similar study confirmed that the WHC of BC membrane was at least 106.43 folds its dry weight [23]. Another study described the WHC of BC membrane that was at least 65.6 folds of it is own weight [24]. The variations in WHC of these BC membranes can be attributed to their respective porosities and surface areas [23] (Table 1).

Antibiotics loading capacity

Due to the unique properties of BC membrane, studying the absorption of antibiotics at different concentraions from BC revealed interesting information about the characteristics of the BC. The relationship between different antibiotic concentrations and the uploading capacities of BC membranes were represented in (Table 2).The immersion of BC membranes in different concentrations of antibiotics were carried out for 24 hours. As the four concentrations of each antibiotic were rather low, the drug-uploading capacities of BC membranes increased almost linearly with the rise of antibiotic concentrations. It was concluded that, as the concentration of antibiotics in the solution increased, more antibiotic molecules were observed to diffuse and absorb into the BC membrane. The present results were in agrement with other studies [14, 25, 28]. From data demonastrated in Table 2, it can be seen that the antibiotics uploading capacities of BC membrane at different concentrations were higher in GE followed by AM and then CH.

Antibacterial activity

BC membrane was efficiently impregnated with antibiotics [14, 28, 33, 34]. Four different concentrations (0.025, 0.050, 0.075 and 0.100 mg/ml) from the antibiotics (GE, AM and CH) were loaded on different BC membranes (1cm^2) each. BC-LA were investigated for their antibacterial activities at different concentrations by the disc diffusion method and the results were represented in Table 3. It was found that all BC membranes prepared under different concentrations of CH exhibited antibacterial activities against the four models of bacteria. While BC membrane loaded with different concentrations of AM exhibited positive antibacterial activities against *E. coli, P. aeruginosa and B. subtilis*. BC-AM membrane exhibited inactive antibacterial activities against *S. aureus*. BC-GE membrane at different concentrations exhibited positive antibacterial activities against *P. aeruginosa* and *B. subtilis*, with no antibacterial activities against *E. coli* and *S. aureus*. Based on the diameters of inhibition zones against the four models of bacteria, as showed in Figure1, the higher antibacterial activities were observed for both *B. subtilis* and *P. aeruginosa*, followed by *E. coli* and then *S. aureus* for BC-CH membrane. The higher antibacterial activities were observed for *P. aeruginosa*, followed by *B. subtilis* and then *E. coli* for BC-AM membrane and the higher antibacterial activities were observed for *P. aeruginosa*, followed by *B. subtilis* for BC-GE membrane. The BC-GE and BC-AM membranes exhibited highest antibacterial activity on *P. aeruginosa* than the other used models of bacteria [24]. BC-CH membrane displayed highest antimicrobial activity on *S. pneumonie* followed by *S. aureus* and *E. coli,* while the present study reported that the highest antibacterial activity was displayed on *P. aeruginosa* followed by *B. subtilis* and then *E. coli* when BC-CH membrane was used [35]. From the data shown in Table 3, the higher concentration of

antibiotics (GE, AM and CH) brought about the higher antibacterial activity, these results suggested that the BC membrane was characterized by a high adsorption capacity. As expected, no inhibition zones were observed for pure BC membrane, implying that BC membrane did not have any antibacterial properties against the four models of bacterial strains (Figure 1). It could be concluded that the antibacterial activity was only attributed to the antibiotics impregnated inside the BC membrane and not the BC membrane itself [14, 22].

Characterization of pure BC and BC-LA membranes

The morphologies structure of BC membrane as a control and the prepared BC-LA (0.025 mg/ml) were investigated using SEM (Figure 2). Figure 2 showed the morphology of BC that exhibited a nonporous network structure with a random arrangement of ribbon-shaped microfibrils without any preferential orientation. The pure BC membrane showed a high density of pores spread over the surface of the BC membranes which were held with the loaded antibiotics [36]. In case of BC-LA membranes, the antibiotic particles were displayed as white spots in the composite membranes and the BC-LA appeared less porous than pure BC which was illustrated in Figures 2B, C and D for GE, CH and AM respectively.

The chemical structure and functional group of different BC membranes were identified by FT-IR at wave numbers ranging from 500 to 4000 cm^{-1} as well as the interaction between BC membrane and loaded antibiotics were identified by comparing FT-IR spectra of BC membrane and BC-LA at concentration 0.025 mg/ml (Figure 3). The band of intense absorption in BC spectrum at wave number 3352.6 cm^{-1} was attributed to the presence of hydroxyl group (OH) of cellulose type I which was in agreement with [37] and [38].At below 2000 cm^{-1}spectrum, GM was observed [39]. The significant amide bands of GE were performed at 1653 cm^{-1} and 1558 cm^{-1} [40] Characterization of BC-GM membrane was identified by comparing the RT-IR spectra of pure BC and BC-GM membrane. The new band that was observed at 1539 cm^{-1} in BC-GM membrane spectra was amine groups (N-H bending) for GM. This proved that BC membrane contained GM [24, 36]. BC-AM membrane was observed at 3325 cm^{-1} and contained amine group (N-H bending) and at 1690 cm^{-1}. C=O was observed from the β-lactam and amide carbonyl group. This proved that the BC membrane contained AM [24]. After the interaction between BC membrane and CH, the stretching bond observation of nitro group appeared at 1569 cm^{-1}. This band indicated that BC membrane contained CH. Nearly, a stretching bond of nitro group at 1563 cm^{-1} was observed due to the combination between CH and BC membrane [35] (Figure 3).

TGA was performed to determine the water retention property and thermal decomposition behavior of BC and BC-LA membranes. Thermal stability of the membranes might be important for some applications and might provide some clues on BC fiber interactions [41]. Figure 4 showed the TGA degradation curve of BC and BC-LA membranes. Three mass loss stages were observed during thermal analysis of the sample. The first stage which occurred at 25 –100 °C contributed to the evaporation of residual water present in BC and BC-LA membranes that showed a weight loss of 5.6, 28.2, 24.4 and 42.03% for pure BC, BC-GE, BC-CH and BC-AM membranes respectively with significant differences between BC samples in this region. This indicated that there were some differences in the moisture contents between these samples due to their loading with antibiotics while pure BC exhibited a low mass loss because no antibiotics were loaded on it. The second stage that occurred in the range of 200–400 °C was associated with a series of reactions (degradation of cellulose) including dehydration, depolymerization of the glycoside units and decomposition of glucose units followed by the formation of a carbonaceous char [42]. This second mass-loss stage contributed to the higher loss of mass of pure BC than BC-LA membranes (56.2, 41.3, 43.4 and 33.07% a weight loss of pure BC, BC-GE, BC-CH and BC-AM membranes respectively) which was characterized by the onset temperature (T_{Onset}) [43]. The third stage that occurred above 450 °C showed the oxidation and breakdown of carbonaceous residues yielding gaseous products of low molecular weight and this was known as the carbonaceous stage [43, 44]. TG analysis in Figure4 confirmed that BC and BC-LA membranes were very stable and had no degradation up to 250 °C.

Biological studies

Macroscopic examinations of wound areas and measurements of wound area closure

The results of the present study demonstrated wound sites (Figure 5) of all animals and the percentages of wound area closure at 3, 7 and 14 days (Table 4). The percentages of wound closure in the wounded group (W) were minimal 1.64% and 7.15% after 3 and 7 days of wound induction which progressed to record 26.91% after 14 days of study period. On applying BC membrane on wound area, the percentages of wound closure

began to increase recording 5.47, 10.43 and 41.03% at 3, 7 and 14 days respectively. Applying BC-GE membrane revealed 23.75% of closure against 18.71 and 9.04 % of BC-AM and BC-CH respectively after 7 days of wound induction. These percentages of wound healing increased after 14 days of applying different BC-LA membranes to record 76.58,58.66 and 41.69 in wounded rats treated with BC-GE, BC-AM and BC-CH respectively (Figure 5 and Table 4) revealing the biological wound closure activity of BC-GE membrane. BC and BC-LA membranes adhered to wound sites very well by their elastic properties which allowed an excellent molding to the wound site [1].The applied never-dried BC membranes allowed maintenance of a proper moist environment around the wound with absorbance of the wound's exudates [45]. This accelerated the entire process of re-epithelialization in comparison with the uncovered wounded in W group of rats [1]. The presence or easy access of pathogenic bacteria in different wounds can hinder their healing [46]. BC membrane possesses physical and mechanical properties that accelerates healing [1] but it lacks antibacterial effects resulting in its failure to provide a barrier against wound infection. [14] Furthermore, the current therapies to treat bacterial infection in wound area rely on systematic administration of antibiotics risking systemic toxicity or topical pharmaceutical formulations of ointments that rapidly absorb fluid from the wound area [47]. Here in this study the biologically investigated BC-LA membranes were prepared using the smallest antibiotic concentrations. These results were in accordance with Elsner et al. [48] who evidenced that treating wounds by BC-LA membranes may be preferred due to the reduction of drug doses hence systemic toxicity, low occurrence of bacterial resistance, drug chemical stability in the presence of BC and controlled release with no need for frequent wound healing membrane changes. Likewise, the release of antibiotics from BC membranes suggests that BC membranes possess bio adhesive properties and ability to provide sustained drug release [49]. BC-GE membrane achieved the best closure properties in wound area. BC-GE membrane had absorbed and adsorbed GE effectively and released it effectively and gradually for 14 days. GE is encapsulated into hollow nanofibers of BC membrane resulting in a better control of antibiotic release profile resulting in the best wound closure activity [50] (Table 4 and Figure 5).

Wound healing parameters

Wound healing occurs in four processes which includes hemostasis, inflammatory, proliferative or fibroblasts phase (that includes collagen synthesis, neovasculature formation and re-epithelialization) and wound contraction [7]. For a good wound healing formulation, it must contain active ingredients, which possess good aqueous solubility, better tissue absorption, delayed metabolism and long plasma half-life with establishment of new blood vessels [51]. Inflammatory cytokines activate endothelial cells attracting neutrophils and macrophages. Resident endothelial cells respond to number of angiogenic factors, including VEGF [52]. Consequently, angiogenesis is stimulated in BC-treated groups at all-time points resulting in neovascularization [15]. The present study elucidated a significant ($p < 0.05$) increase in wound tissue VEGF, fibronectin when applying BC membrane for 14 days (Table 5). In addition, applying BC-LA membranes showed a significant ($p < 0.05$) enhancement of VEGF that was clearly manifested in W/BC-GE treated rats. During proliferative or fibroblasts phase, extracellular tissue matrix proteins are synthesized and deposited, such as fibrous proteins, collagen, polysaccharides, fibronectin and proteoglycans. Fibrin and fibronectin cross link together and form the main structural support for the wound until collagen is deposited [53].Collagen produced by fibroblasts is the most important constituent of connective tissue that confers strength and integrity to the tissue matrix and plays an important role in epithelialization at the latter phase of healing [54]. Out of the many subtypes of collagen, type I collagen is mostly seen in healing tissues [51]. In addition, the measurement of hydroxyproline, which is produced by the breakdown of collagen is used as an index of collagen turnover in wound site [55]. In the present study, there was a significant ($p < 0.05$) decrease in wound tissue collagen I, hydroxyproline and total protein content in W group compared to NC group. Collagen synthesis and consequently hydroxyproline and total protein noticeably decreased causing chronic connective tissue complications and defective wound repair in W group (Table 5). Applying BC membrane either alone or loaded with different antibiotics to the wounded area produced a significant ($p < 0.05$) increase in wound tissue collagen I, hydroxyproline and total protein when compared to W group [56]. This elevation was clearly manifested in BC-GE group when compared to W or W/BC groups. However, these parameters began to increase after covering wound areas by BC and BC-LA membranes. Findings of this study were consistent with earlier studies that proved that higher hydroxyproline levels at day 14 of wound induction content reflected increased cellular proliferation [54] and indicated good healing nature of different BC-LA membranes as they enhanced the production of collagen fibers and wound strength which is involved with better wound healing [57]. MPO activity was studied to evaluate the presence of polymorphonuclear neutrophils in the wounded skin. Wound tissue inflammation leads to a situation known as oxidative burst in which the consumption of oxygen dramatically increases [58]. This provides

favorable conditions for the formation of oxygen-derived free radicals by means of leukocytes, mostly neutrophils. The persistent infiltration and activation of neutrophils may lead to an abnormal repair response that has profound effects on reducing cell migration, matrix deposition and angiogenesis [59]. The current study clarified a significant ($p < 0.05$) increase in wound tissue MPO in W group compared to NC group. BC and BC-LA membranes decreased wound tissue MPO after 14 days by .72, 0.75, 0.76 and 0.80 in W/BC, W/BC-GE, W/BC-AM and W/BC-CH respectively. This may be due to the reduced neutrophil infiltration and lipid peroxidation inhibition, which in turn leads to a reduction in the formation of the chemotactic intermediates [13]. Additionally, the increased bacteria in wound area responding to infection is another factor in delayed wound healing [58]. *In vitro* antibacterial effects of this study that was carried out against Gram-negative and Gram-positive were consistent with the biological findings that BC-LA membranes served as wound healing membranes in decreasing inflammation.

Inflammatory parameters

A significant increase was recorded in the mean values of plasma ICAM-1, TNF-α and IL-6 after 7 days of wound induction recording 2.9,3.2,2.3 folds then it began to decrease gradually after 14 days of wound induction to record 2.4,2.86 and 1.81 folds increase in the pre-mentioned plasma parameters respectively in W group compared to NC. After 7 days of applying the BC membranes to different wound areas in W/BC group, plasma ICAM-1, TNF-α and IL-6 recorded a significant ($p < 0.05$) increase that began to decline after 14 days in the same group compared to W group (Table 6). The continuous porous structure of BC membrane provided a moist and highly hydrophilic environment, as well as mechanical strength around the wound during the healing process [2]. One of the major concerns of using BC membranes alone in wound areas is the un-degradable or slow degradable properties of BC that could induce chronic inflammation and foreign body reaction [60]. In addition to bacterial infections in wound areas that have a tremendous health and economic impact and they are a threat to patients [61]. Herein, we proposed a different strategy that can overcome the bacterial infections in wound areas using BC membranes loaded with different antibiotics. Loading CH to BC membrane for 7 days in W/BC-CH group verified 0.53, 0.57 and 1.98 folds decrease in ICAM-1, TNF-α and IL-6 respectively compared to W group. The decrease in the mean values of ICAM-1, TNF-α and IL-6 continued for 14 days in W/BC-GE to record 0.48,0.54 and 0.67 folds decrease respectively compared to W group. In addition, applying BC-AM membrane to rats' wound areas for 7 days produced 0.63, 0.69 and 0.70 fold decrease in the pre-mentioned parameters respectively. This decrease continued with slight changes after 14 days of applying this membrane to record 0.63, 0.66 and 0.79 in ICAM-1, TNF-α and IL-6 respectively compared to W group. Applying BC-GE membranes to rat wound site for 7 days induced 0.72,0.77 and 0.75 fold decrease in the mean values of ICAM-1, TNF-α and IL-6 respectively. By the progress of time for 14 days this decrease changed slightly recording 0.72, 0.76 and 0.83fold decrease when compared to W group (Table 6). TNF-α and IL-6 are effective cytokines which are mediators of the body's response to infection and key regulators of inflammatory responses. They are thought to impair collagen accumulations and inhibit the proliferative phase of wound healing [62]. Furthermore, intercellular adhesion molecule-1 (ICAM-1) is central to the inflammatory process. ICAM-1 is present on endothelial cell membranes and is essential to leukocyte adhesion and migration during inflammation [63]. During wound repair, the inflammatory response occurs where recruitment of cells is done to act against potential bacterial contamination of the wound and stimulates cytokine secretion to activate the processes of dermal and epidermal repairing [64]. The present data confirmed that wound healing properties were increased using BCLA membranes that led to the reduction in inflammation (Table 6).Previous literature also indicated that during the early phase of wound repair, pro-inflammatory cytokines are predominantly expressed in polymorphonuclear leukocytes and are reduced during progression of wound healing and the rise of cytokine levels was later reduced to the optimum level on progression of healing process.

ISSN: 0975-8585

Figure 1. Optical images of inhibition zones of BC-LA and pure BC membranes: *E. coli* (A), *P. aeruginosa* (B), *B. subtilis* (C), *S. aureus* (D) and pure BC membrane as a control (E). In all plates, (a-d) are BC-0.025, BC-0.050, BC-0.075 and BC-0.100 mg/ml respectively.

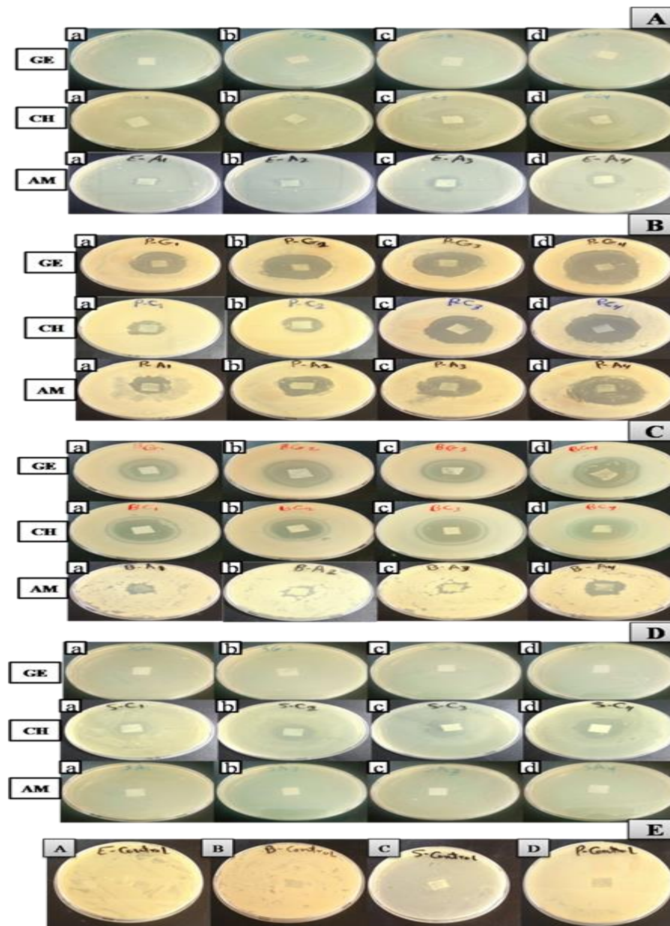

Figure 2. SEM morphology of (A) pure BC, (B) BC-GE, (C) BC-CH and BC-AM membrane at concentration 0.025 mg/ml.

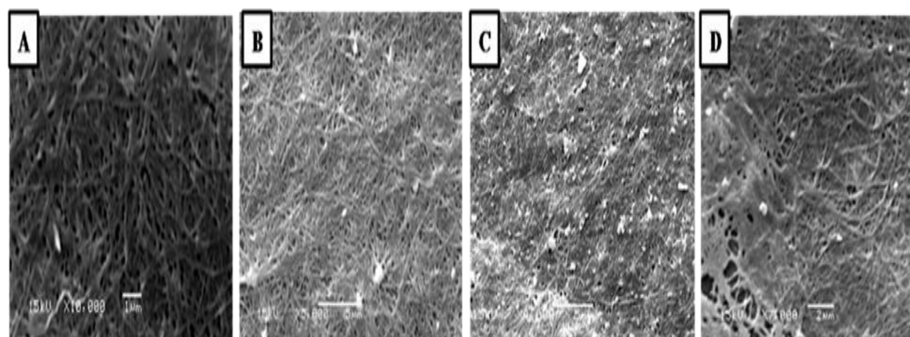

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Figure 3. FT-IR spectra of pure BC and BC-LA membrane (GE, CH and AM) at concentration 0.025 mg/ml.

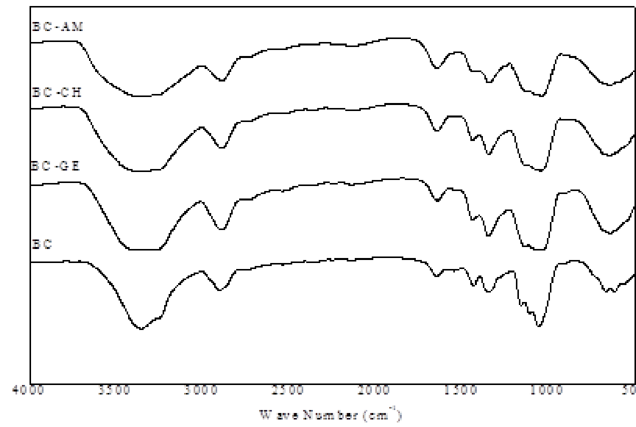

Figure 4. TGA analysis of pure BC and BC-LA membrane (GE, CH and AM) at concentration 0.025 mg/ml.

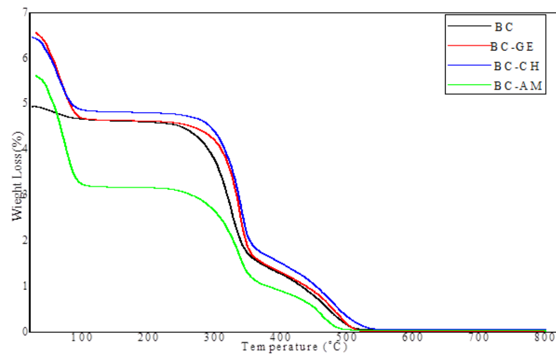

Figure 5. Wound closure after 3, 7 and 14 days in experimental groups

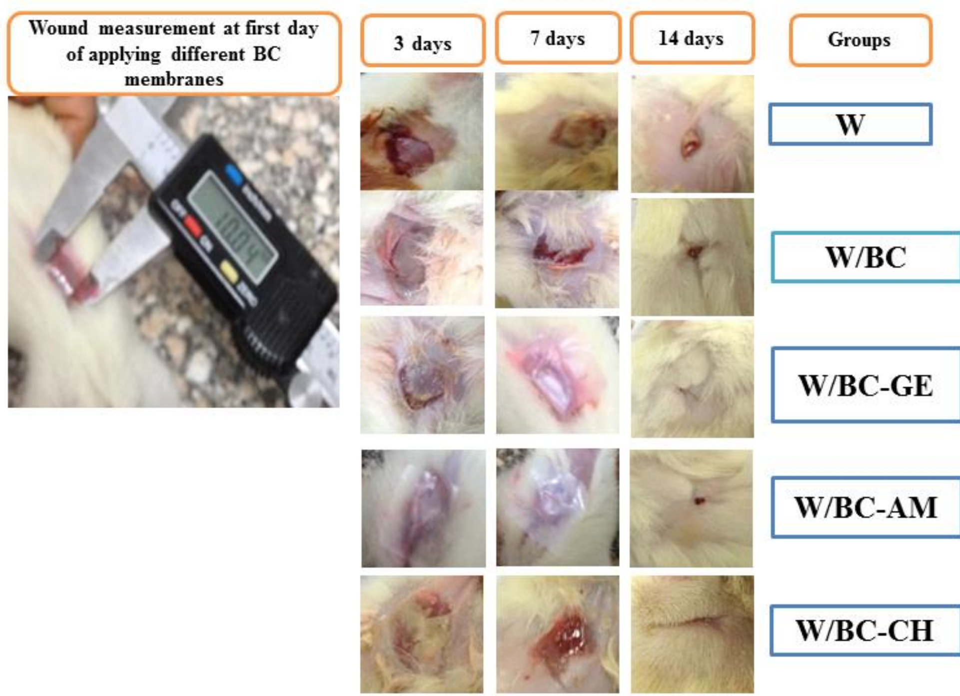

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Table 1: Water holding capacity of BC membrane (WHC).

Samples	Weight of dry BC membrane	Weight of hydrate BC membrane	WHC (mg/cm^2)
1	0.0019	0.0069	263.1
2	0.0023	0.0073	217.3
3	0.0019	0.0070	268.4
4	0.0028	0.0078	178.5
Average			231.8

Table 2: Antibiotic loading capacities of BC membranes at different concentrations for GE, CH and AM.

Antibiotics	Antibiotics concentration (mg/ml)	Absorbed antibiotics on BC membrane (mg)	Antibiotics upload capacity (mg/cm^2)
GE	0.025	0.0074	0.0074
	0.050	0.0083	0.0083
	0.075	0.0094	0.0094
	0.100	0.0103	0.0103
CH	0.025	0.0021	0.0021
	0.050	0.0026	0.0026
	0.075	0.0033	0.0033
	0.100	0.0045	0.0045
AM	0.025	0.0025	0.0025
	0.050	0.0031	0.0031
	0.075	0.0038	0.0038
	0.100	0.0049	0.0049

GE: Gentamicin; CH: Chloramphenicol; AM: Ampicillin

Table 3: Diameter of inhibition zone of BC–LA at different concentrations against E. coli, P. aeruginosa, B. subtilis and S. aureus, and (original diameter of BC membrane was 10 mm).

Organism	Antibiotic concentrations (mg/ml)	Diameters of inhibition zone (mm)		
		GE	CH	AM
E. coli	0.025	0	30	16
	0.050	0	31	19
	0.075	0	32	22
	0.100	0	35	24
P. aeruginosa	0.025	38	24	28
	0.050	43	29	33
	0.075	47	43	38
	0.100	51	44	45
B. subtilis	0.025	38	41	19
	0.050	39	42	25
	0.075	41	44	27
	0.100	44	45	29
S. aureus	0.025	0	2.2	0
	0.050	0	2.3	0
	0.075	0	2.6	0
	0.100	0	2.8	0

GE: Gentamicin; CH: Chloramphenicol; AM: Ampicillin

Table 4: Mean ± SEM of wound closure percentages in wound areas in differential experimental groups

	3 days	7 days	14 days
W	1.64 ±0.21	7.15±0.12	26.91 ±0.17
W/BC	5.47 ^{b} ±0.14	10.43^{b}±0.15	41.03 ^{b}±0.23
W/BC-GE	16.55^{b}±0.18	23.75^{b} ±0.67	76.58^{b}±0.12
W/BC-AM	8.53 ^{b} ±0.28	18.71 ^{b} ±0.13	58.66^{b} ±0.13
W/BC-CH	2.47 ±0.20	9.04^{b}±0.13	41.69^{b} ±0.28

W: wounded without BC membrane; W/BC: wounded treated with bacterial cellulose; W/BC-GE: wounded and treated with bacterial cellulose-Gentamicin; W/BC-AM: wounded and treated with bacterial cellulose-Ampicillin; W/BC-CH: wounded and treated with bacterial cellulose-Chloramphenicol.
^{b} $p < 0.05$ compared to W group.

Table 5: Means ± Standard error of means of tissue Collagen I (pg/mg tissue), Hydroxyproline (ng/mg tissue), Fibronectin (ng/mg tissue), VECGF (Pg/mg tissue), MPO (ng/mg tissue) and total protein (µg/mg tissue) after 14 days of wound induction

	Collagen I	Hydroxyproline	Fibronectin	VEGF	MPO	Total protein
NC	724.34±5.70	53.9 ±0.10	11.31 ±0.11	669.58 ±4.91	1.26 ±0.03	87.31 ±1.80
W	445.80 ^{a} ±5.95	22.5 ^{a} ±0.01	6.70 ^{a} ±0.01	259.04 ^{a} ±3.03	3.71^{a} ±0.03	53.73 ^{a} ±0.73
W/BC	490.40^{ab}±5.12	29.0 ^{ab}±0.04	7.99 ^{ab}±0.05	338.55 ^{ab}±8.28	2.69 ^{ab}±0.01	73.17 ^{ab}±1.57
W/BC-GE	697.66^{abc}±4.62	49.0 ^{abc}±0.03	9.69 ^{abc}±0.01	598.50 ^{abc}±2.99	2.78 ^{abc}±0.02	78.74 ^{abc}±2.56
W/BC-AM	656.90^{abc}±7.40	40.5 ^{abc}±0.06	9.03 ^{abc}±0.03	535.27 ^{abc}±4.37	2.85^{abc}±0.03	65.04 ^{abc}±0.95
W/BC-CH	558.20^{abc}±5.46	34.7 ^{abc}±0.03	8.52 ^{abc}±0.04	469.93 ^{abc}±5.27	3.00 ^{abc}±0.02	63.16 ^{abc}±1.49

NC: Normal control without wound; W: wounded without BC membrane; W/BC: wounded treated with bacterial cellulose; W/BC-GE: wounded and treated with bacterial cellulose-Gentamicin; W/BC-AM: wounded and treated with bacterial cellulose -Ampicillin; W/BC-CH: wounded and treated with bacterial cellulose-Chloramphenicol. ^{a} $p < 0.05$ compared to NC group, ^{b} $p < 0.05$ compared to W group, $^{c}p < 0.05$ compared to W/BC group.

Table 6: Means ± Standard error of means of Plasma ICAM-1, TNF-α and IL-6 (pg/ml) after 7 and 14 days of wound induction in experimental groups.

	7 days			**14 days**		
	ICAM-1	TNF-α	IL-6	ICAM-1	TNF-α	IL-6
NC	848.77±4.21	4.34±0.03	10.34±0.07	857.94±4.62	4.33±0.01	10.40±0.03
W	2477.90 ^{a} ±11.56	14.24±0.20 ^{a}	24.26 ^{a} ±0.54	2085.70^{a} ±13.47	12.42 ^{a} ±0.09	18.83 ^{a} ±0.07
W/BC	2182.50 ^{ab} ±8.22	12.95^{ab}±0.21	20.57 ^{ab}±0.28	1891.72 ^{ab} ±7.57	10.87^{ab}±0.06	17.61 ^{ab} ±0.08
W/BC-GE	1786.50 ^{abc} ±9.50	11.09^{abc}±0.11	18.23^{abc} ±0.09	1509.82^{abc} ±12.61	9.51 ^{abc} ±0.12	15.80 ^{abc} ±0.11
W/BC-AM	1578.10 ^{abc} ±12.55	9.93 ^{abc} ±0.12	17.17^{abc} ±0.19	1315.11^{abc} ±13.58	8.23 ^{abc} ±0.13	14.98 ^{abc} ±014
W/BC-CH	1326.90^{abc}±13.00	8.18 ^{abc} ±0.09	15.14^{abc}±0.09	1013.00^{abc}±17.35	6.76 ^{abc} ±0.07	12.72 ^{abc} ±0.05

NC: Normal control without wound; W: wounded without BC membrane; W/BC: wounded treated with bacterial cellulose; W/BC-GE: wounded and treated with bacterial cellulose -gentamicin; W/BC-AM: wounded and treated with bacterial cellulose -Ampicillin; W/BC-CH: wounded and treated with bacterial cellulose-Chloramphenicol. ^{a} $p < 0.05$ compared to NC group, ^{b} $p < 0.05$ compared to W group, $^{c}p < 0.05$ compared to W/BC group.

CONCLUSION

The current study indicated that the impregnation of gentamicin, ampicillin and chloramphenicol on BC membranes as extracellular product synthesized by *Komagataeibacter hansenii* introduced antimicrobial activities into bacterial cellulose membrane. In addition, the biological investigation of BC and BC-LA at minor concentration showed enhanced wound healing and tissue regeneration activity in wounded rat model. Particularly BC-GE membrane elucidated the most ameliorative wound healing potency in wounded rat model. Because of these findings, it is suggested that these BC-GE membrane could be considered as potential dressing material after further clinical evaluations.

ACKNOWLEDGEMENT

The authors extend their appreciation to Al-Azhar Center for Fermentation Biotechnology and Applied

Microbiology and to Prof. Dr: Ayman A. Farrag the manager of this Center for supporting this work.

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