The healing property of a bioactive wound dressing prepared by the combination of bacterial cellulose (BC) and *Zingiber officinale* root aqueous extract in rats

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Abstract

Gluconacetobacter xylinus was used for production of bacterial cellulose (BC). The obtained BC was washed and floated in the nontoxic dose of the herb aqueous extract that was obtained from the methyl thiazol tetrazolium (MTT) assay. Twenty-four Wistar rats were divided into four separated groups and after inducing the wounds (15 mm in diameter), each group was treated with honey, BC, herb aqueous extract, and the combination of BC–herb aqueous extract. Each day the contraction percentages of the wound sites were measured. On days 3, 7, and 14, two rats from each group were euthanized, the skin samples from the wound regions were achieved and their paraffin blocks were prepared. Finally after trichrome staining, the microscopic examinations were done. MTT assay results indicated that the herb aqueous extract had dose-dependent toxic effects and the nontoxic dose of the extract was prepared and utilized for in vivo assay. Although the macroscopic analysis revealed that the BC and the herb aqueous extract had better activity in the wound contraction percentages than their combination, microscopic analysis indicated that the combination of BC–herb aqueous extract revealed all the characteristics that each material induced in the wound site alone. In conclusion, the speed of the wound healing should not be solely considered and its quality should be considered as well.

Keywords Bacterial cellulose (BC) · *Zingiber officinale* root · Aqueous extract · Wound-healing assay

Introduction

A wound refers to an injury that damages the skin and then the regeneration process will be started (Sen et al. 2009). To accelerate and control this process, it is recommended to manage the wound site with an appropriate wound dressing. The aim of the utilization of the wound dressing is to retain a moist environment and cover the wound area to prevent the entry of the pathogenic microorganisms into the wound site. Also wound dressing aids to increase the inflammation, angiogenesis, and collagen bundles' formation (Bishop et al. 2003). In the classification of different wound dressings based on their action, there are three main categories which are named passive, interactive, and active dressings (Pilehvar-Soltanahmadi et al. 2018). As it was mentioned

above, the main aim of the dressing is to cover the wound surface and protect it from the invasion of the microorganisms. This primary aim is achieved by utilizing the passive type of wound dressing such as Paraffin and sterile gauzes (Pilehvar-Soltanahmadi et al. 2018). If the used dressing in addition to its covering and protection function helps to maintain the water content and transfer of oxygen to the wound region, this type of dressing is named the interactive one (Pilehvar-Soltanahmadi et al. 2018). The examples of this group are foams and polymeric films that among their advanced forms are hydrocolloids, hydrogels such as alginate, and hydrofibers. Finally, if the used dressing in addition to the above characteristics contains some drugs and delivers them to the wound region then it is named the bioactive type. In the mentioned category there are some biological dressings such as microbial alginate, gelatin, chitosan, bacterial cellulose (BC), collagen, hyaluronic acid (HA) and many other types of biopolymers (Pilehvar-Soltanahmadi et al. 2018).

Cellulose is the natural polymer with β (1, 4)-linked D-glucose which has high water maintenance and fine

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microfibrillar mesh structure that acts as a microbial barrier. It was reported that cellulose is nontoxic and biocompatible with the human skin. This water-insoluble polymer can be extracted from plants and surface of some bacterial culture media (Pourali et al. 2014). It was indicated that some types of bacterial genera which belong to the Gram-negative ones can produce BC in aerobic and anaerobic conditions (Ji et al. 2016). Among these genera are *Gluconacetobacter*, *Alcaligenes*, *Acetobacter*, *Salmonella*, *Pseudomonas*, *Rhizobium*, *Sarcina*, *Agrobacterium*, and *Aerobacter* (Esa et al. 2014; Kucińska-Lipka et al. 2015). Although cellulose has an acceptable wound-healing activity, the combination of this polymer with some natural and traditional substances such as some types of herb extracts with anti-inflammatory, antioxidant and/or anti-microbial activities made it a good candidate for wound-healing purposes. Based on the herb type, its leaf, bark, and root can be utilized (Pilehvar-Soltanahmadi et al. 2018). Ginger with the scientific name of *Zingiber officinale* is among the medicinal plants with wound-healing activity. More than 400 different chemical components were extracted from *Z. officinale* which indicated different properties and only a few of them were studied carefully (Grzanna et al. 2005). One of the most significant properties of the mentioned herb is its anti-inflammatory effect (Grzanna et al. 2005).

It was observed that *Z. officinale* has anti cytokine activity and can upregulate the genes of the pro inflammatory cytokines (Grzanna et al. 2005).

Ginger has anti-nausea, anti-thrombotic, and anti-migraine properties. Some ginger components have serotonin receptor blocking activity (Al-Amin et al. 2006). There are some studies about the wound-healing property of the rhizome of *Z. officinale* (Goel and Sairam 2002; Penna et al. 2003; Ojewole 2006; Bhagavathula et al. 2009).

Although BC was successfully applied as a wound dressing material in the wound region, (Kucińska-Lipka et al. 2015), there is no available report on the histological alterations of the wound region after being treated with the altered BC, which is impregnated by herb extract to produce a new type of bioactive wound dressing. Hence, the current research was conducted to evaluate the wound-healing efficiency of the BC (as a type of bioactive dressing) that was impregnated with aqueous extract of *Z. officinale* (as a type of traditional dressing) for treatment of the rat cutaneous wounds.

Materials and methods

Culturing of the BC-producing bacterium

Gluconacetobacter xylinus (PTCC 1734) was purchased from Iranian Biological Resource Center (IBRC) and

cultured in Hestrin–Schramm (HS) broth medium. The flask was incubated at 30 °C for 8 days. The appearance of the yellowish film on the top of the broth medium indicated the production of the BC (Pourali et al. 2018).

The purification of the BC film

The produced BC film contained some impurities such as the used bacterial strain that was trapped in the layer. Hence the obtained BC membrane was floated overnight in 3% NaOH solution and was washed by ddH₂O until the pH of the membrane reached around 7.3–7.5 (Pourali et al. 2014).

Confirming the BC production

To confirm the obtained layer was BC, after washing the film it was air-dried and utilized for enzymatic hydrolysis. 0.05 mL of two types of endo glucanase and exo glucanase enzymes (0.5 U, Sigma-Aldrich, USA) in 0.2 mL of sodium acetate buffer (0.05 M, pH 5.5) were added to the BC layer, and after incubation at 30 °C for 24 h in the shaking condition (50 rpm), by the use of Molisch's and Benedict's reagents the presence of the carbohydrates and reducing sugars were confirmed, respectively (Pourali et al. 2014).

Preparation of the aqueous extract of *Z. officinale*

The fresh root of *Z. officinale* was collected and rinsed with the tap water and ddH₂O. The herb root was dried at room temperature for 10 days and using an electric grinder it was completely powdered. To prepare the herb aqueous extract, 5 g of the herb powder was dissolved in 100 mL of ddH₂O and heated at 100 °C for 30 min. The extract was filtered using Whatman filter and the extract was preserved at 4 °C before further experimentation (Siddhan et al. 2016).

In vitro assay

Before the in vivo assay it was important to obtain the toxic and nontoxic doses of the herb extract. Therefore methyl thiazol tetrazolium (MTT) assay was utilized (Pourali et al. 2017). For this aim, the herb aqueous extract was sterilized using tyndallization technique. Briefly, in three continuous days the sample was heated indirectly using the Bain-marie for 30 min and incubated at 37 °C for 24 h. Mouse fibroblast cell line (NIH-3T3 D4) that was purchased from the Pasteur Institute of Iran was used for MTT assay. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma-Aldrich, USA) that was enriched by 10% fetal bovine serum (FBS, Sigma-Aldrich, USA), and 1% penicillin–streptomycin (Sigma-Aldrich, USA). Two rows of a 96-well micro titer plate that were seeded by the cells were filled with 200 µL of the working medium. The plate

was incubated at 37 °C for 24 h in the presence of 5% CO₂. In the next day the test was started from the second well in the second row of the plate. 200 µL of the herb aqueous extract was added to this well and after pipetting, 200 µL of it was transferred to the third well and this titration was continued until the tenth well. Finally, 200 µL of the tenth well was removed. 11th well was used as control which solely contained 200 µL of the working medium. For the test repeat, the third row was used and the above procedure was repeated. The plate was incubated at 37 °C for 24 h in the presence of 5% CO₂ and in the next day the mixture was removed. The surface of the cells was washed using phosphate-buffered saline (PBS) and the wells were filled with 20 µL of 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as MTT (Sigma-Aldrich, USA) dye solution. After incubation at 37 °C for 4 h in the presence of 5% CO₂ the dye was removed and 100 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, and USA) was added to all the wells. The plate was placed in the shaker incubator at 37 °C for 20 min and the optical densities (ODs) of the wells were achieved at 580 nm by the aid of ELISA reader instrument (Pourali and Yahyaei 2018). Finally, the half maximal inhibitory concentration (IC₅₀) value which means the ability of the herb extract in inhibiting the cell proliferation was determined. The well which was before the determined IC₅₀ well was considered as toxic and the well after the determined IC₅₀ well was considered as the non toxic doses, respectively. The cell viability percentages were determined using the obtained OD values. Finally, the BC layer was floated overnight in the determined non toxic dose of the ginger aqueous extract and was used for in vivo assay (Pourali et al. 2016).

In vivo assay

Twenty-four Wistar rats (female, 180 g weight, 4 weeks old) were purchased from Pasteur Institute of Iran and were kept in separated cages 10 days before starting the experiments, with 12 h light and 12 h dark cycles, and were given food and water *ad libitum*. The rats were divided randomly in four separated groups ($n=6$): three control groups; one of them was treated with BC and the layer was moistened with 0.2 ml of ddH₂O daily, the other one was treated with 0.2 ml of herb aqueous extract daily and the third group received 0.2 ml of honey daily. The test group was treated with the combination of BC–herb aqueous extract and the layer was moistened with 0.2 ml of herb aqueous extract daily. The duration of the experiment was 14 days. After anesthizing, shaving, and disinfecting the skin dorsal surface of each rat, a circular wound with the full thickness (15 mm in diameter) was created for the experiment. Each group was treated by the above-mentioned materials and the sizes of the open wounds were measured daily. The wound contraction

percentages (%) were calculated using the formula: [size of the open wound (mm) in the first day-test day]/size of the open wound (mm) in the first day] × 100. The resulted data were compared to each other by employing version 22 of SPSS software and p value < 0.05 was considered as significant (Pourali and Yahyaei 2016).

Finally, on days 3, 7, and 14 two rats from each group were euthanized and full thickness of the skin samples with sizes of 2 × 2 cm² from the wound regions was achieved and fixed using 10% formalin. The samples were embedded in the paraffin and the 5 µm diameter sections of the achieved blocks were prepared by the aid of microtome apparatus. The sections were transferred to the microscopic slides and were subjected to the trichrome-staining method. Microscopic examination was performed and the factors such as levels of the collagen bundles formation, the infiltration of the inflammatory cells, reepithelization, fibroplasia, and angiogenesis were compared to each other (Hegggers et al. 1996; Pourali and Yahyaei 2016).

Results

Culturing of the BC-producing bacterium

G. xylinus was cultured in HS broth medium and after 8 days of incubation, the yellowish film on the top of the broth medium was achieved. The obtained BC film is demonstrated in Fig. 1.

Purification of the BC film

The produced BC film was floated overnight in 3% NaOH solution and was washed using ddH₂O until the membrane pH reached around 7.3. The membrane was uniform, with high stability.

Fig. 1 The obtained BC film after 8 days of incubation (scale bar = 1 cm)

Confirming the BC production

To confirm the obtained layer was BC, after pre treatments, the membrane was used for enzymatic hydrolysis. The results indicated that after adding the Molisch and Benedict's reagents, the red and orange colors appeared in the solutions, respectively. These changes in the solution color confirmed the presence of carbohydrates and reducing sugars in the products of the enzymatic hydrolysis of the BC membrane, respectively.

In vitro assay

As it was indicated previously, the non toxic dose of the herb aqueous extract should be determined before in vivo assay. MTT assay results of the mouse fibroblast cell line (NIH-3T3 D4) revealed that the herb aqueous extract had dose-dependent toxic effects. The IC_{50} of the sample was in the third well of the plate, so the well after that (i.e., the fourth well) was considered as the non toxic dose. So the herb aqueous extract was diluted 1/8 (which equals to 6.25 mg/ml of the herb powder) and the BC layer was floated overnight in the determined concentration to achieve the combination of BC–herb aqueous extract. Also this non toxic concentration was utilized for the group which was administrated by the herb aqueous extract. Figure 2 indicates the obtained cell viabilities (%).

In vivo assay

As it was mentioned previously, the rats were divided randomly in four separated groups: a group that was treated by BC, a group that was treated by herb aqueous extract, a group that received honey and the last one that was treated by the combination of BC–herb aqueous extract. In 14 days of the experiments the wounds contraction percentages (%) were calculated. Figure 3 demonstrates the wound

macroscopic examination, Fig. 4 indicates the results of the obtained contraction percentages (%) and Table 1 represents the obtained sizes (mm) of the open wounds of four different groups in 3 different days of the treatments (i.e., days 3, 7, and 14).

The obtained results from the one-way ANOVA analysis indicated that there was a significant difference between all the tested groups in day 3. In that day, the group which was treated by the BC had maximum and the group that was treated by honey had minimum contraction percentage. In day 7, there was a significant difference between all the groups except for two groups that were treated by BC and honey. In these two mentioned groups, there was no significant difference and both of them had minimum contraction percentage. The maximum contraction percentage was observed in the herb aqueous extract group. In day 14, there was significance difference between all the groups except for two groups that were treated by the combination of BC- herb aqueous extract and honey. The minimum contraction percentage was observed in these two groups and the maximum contraction percentage was indicated in the BC group.

In the microscopic evaluations, the obtained samples were subjected to trichrome staining method and the parameters such as levels of the collagen bundles formation, the infiltration of the inflammatory cells, re epithelization, fibroplasia, and angiogenesis were evaluated and compared to each other. The microscopic pictures of four different groups in three different days of the treatments (i.e., days 3, 7, and 14) are demonstrated in Fig 5.

As this figure shows, in the group that was treated by BC, in day 3, the amount of fibroplasia and the number of fibroblast cells are appropriate. Low inflammatory cells and low amount of angiogenesis are observed. In day 7, the high amount of fibroplasia and the connective tissue with high contents of thick collagen bundles are present. In addition, the number of inflammatory cells increased remarkably. In day 14, the characteristics of the obtained samples

Fig. 2 The obtained cell viabilities (%). The IC_{50} well was in the third one

Fig. 3 The wound macroscopic examination and the measurement of the size of the open wounds to calculate the contraction percentages (%) of four different groups in 3 different days of the treatments (i.e., days 3, 7, and 14)

Fig. 4 The results of the calculated contraction percentages (%) of four different groups in 3 different days of the treatments (i.e., days 3, 7, and 14)

Table 1 The obtained sizes (mm) of the open wounds of four different groups in 3 different days of the treatments (i.e., days 3, 7, and 14)

Groups	Days		
	3	7	14
BC	11	10	3
Herb aqueous extract	13	5	4
Combination of BC–herb aqueous extract	12	8	5
Honey	15	10	5

are similar to the positive control group. The epithelium is visible along with the thin layer of the stratum corneum. Although collagen bundles appeared, their thickness is not appropriate. Unlike the positive control group, in this group, the hair follicles and fat glands are visible in the dermal part.

In the group that was treated by herb aqueous extract, in day 3, a proper rate of fibroplasia with a large number of fibroblasts and thin collagen bundles are seen. Angiogenesis and inflammatory cell infiltration are noticeably present. In day 7, the severity of angiogenesis and infiltration of the

Fig. 5 The microscopic pictures after trichrome-staining method of four different groups in 4 different days of the experiments ($\times 400$)

inflammatory cells is reduced, and diameter of the collagen bundles is increased. In day 14, epidermis and its epithelium is perfectly formed, and its thickness and stratum corneum is similar to the BC group in that day. Hair follicles and sebaceous glands are abundant in the dermal layer and the thickness of the collagen bundles in the dermal region is increased significantly.

In the group that was treated by a combination of BC–herb aqueous extract, in day 3, the rate of fibroplasia and the number of fibroblast cells are not much increased, and the angiogenesis and newly formed vessels are not observed. The number of inflammatory cells in the wound site is appropriate. Thin and low collagen bundles are also observed. In day 7, the severity of fibroplasia and the number of the connective and inflammatory cells are increased. Collagen bundles have a good thickness, but epidermis and a skin appendage are not seen. In day 14, the epidermis with a suitable thickness is revealed. There is a thin layer of stratum corneum on the epidermis. Collagen bundles have a good thickness and are sufficient. The skin content with a good number and morphology is observed, as well.

In the positive control group that was treated by honey daily, in day 3, the amount of fibroplasia is appropriate,

but collagen bundles are not formed or are in a fine sizes. Angiogenesis and the number of the inflammatory cells are appropriate in the wound environment. In day 7, increasing in the amount of fibroplasia and collagen bundles is present, but, still, the diameter of the collagen bundles is not desirable. The level of angiogenesis and inflammatory cells is still appropriate, but the skin tissue and its appendages are not observed yet. In day 14, the collagen bundles are visible, but they are not thick enough, and the intensity of the angiogenesis and inflammatory cells is reduced. A complete epithelium forms and a thin layer of stratum corneum is revealed at its top.

Discussion

Cellulose is one of the most abundant polymers in the nature that is mainly produced by the plants. Unlike its profusion in the nature, it always has some impurities such as lignin, hemicelluloses, and other components (Ross et al. 1991). The other source of this natural polymer is BC that is produced by some different types of bacterial genera especially *Gluconacetobacter*, and *Acetobacter* groups (Pourali et al.

2018). BC is pure and does not contain the above-mentioned materials and impurities. Its nano-diameter fibers made a microfibril network with high stability and water absorbance ability that makes it a good candidate for some medical applications (Ross et al. 1991). This biopolymer is utilized as a type of bioactive wound dressing that has a high degree of biocompatibility. To have a better activity and wound-healing property of the BC, some drugs or antimicrobial agents such as silver nanoparticles were added to its structure (Pourali et al. 2014; Yahyaei et al. 2016). In a study, Siddhan et al. used *Momordica charantia* and *Tridax procumbens* extracts to enhance the antibacterial efficiency of the BC membrane. They expressed that the BC containing the mentioned herb extracts had acceptable antibacterial properties against some human pathogenic bacterial strains. In this experiment, the new generation of BC was not analyzed in vivo (Siddhan et al. 2016). BC is not solely utilized for wound-dressing applications. For example in a study, some herb extracts were incorporated to the BC with the aim of shelf life extension of the mushrooms (Moradian et al. 2018).

Despite the recent progress in the production of several modern types of the wound dressings, the traditional type of wound coverings such as some herb extracts and animal products have their own attraction (Nayak and Pinto Pereira 2006; Barua et al. 2009; Pilehvar-Soltanahmadi et al. 2018). It is obvious that some types of herb extracts can accelerate the wound-healing process and decrease the possibility of wound infection. This type of dressing, unlike the modern therapeutic products, has low cost and it is usually available due to its presence in a wide range in many regions of the world (Pilehvar-Soltanahmadi et al. 2018). Some herb extracts that are used for wound dressing aims have high water solubility that causes their high bioavailability (Pilehvar-Soltanahmadi et al. 2018). In the present study, the non toxic dose of *Z. officinale* root aqueous extract was applied for treatment of the rat cutaneous wounds. The extract was directly used on the rat skin without any pretreatments such as preparation of its ointment type because it was reported that the ointment types of the medicinal herbs usually have low wound-healing properties due to decreasing the cell-to-cell commutation and suppress the cell migration (Pilehvar-Soltanahmadi et al. 2018).

There are some available reports about the wound-healing efficiency of this traditional herb extract. In the previous study, Bhagavathula et al. analyzed the wound-healing efficiency of two traditional herb extracts; curcumin, ginger extract, and the combination of curcumin–ginger extract. The results revealed that the combination of these two herb extracts had better wound-healing properties than the others (Bhagavathula et al. 2009).

Ginger acts on the metabolic way of prostaglandin synthesis by suppressing two enzymes: cyclooxygenase 1 and

cyclooxygenase 2. This herb can suppresses leukotriene biosynthesis and its act is similar to the anti-inflammatory drugs (Grzanna et al. 2005).

Honey is one of the animal products which is used as a traditional wound dressing from ancient times (Pilehvar-Soltanahmadi et al. 2018). There are some researches about the anti-microbial properties of honey that can eliminate the pathogens from the wound region. This natural substance has anti-oxidant; and anti-inflammatory properties with bactericidal activity (Pilehvar-Soltanahmadi et al. 2018) and in the present research it was used in one of the control groups.

Due to the insufficient researches about the usages of the BC containing some drugs for wound-healing applications, the present research attempted to accelerate the wound-healing properties of the BC and produced the new form of this bioactive wound dressing. For this aim, after the production of BC, *Z. officinale* aqueous extract was incorporated to its tiny microfibril structure and its wound-healing property was evaluated.

The present study employed the pure culture of *G. xylinus* which is known as one of the best BC-producing factories. After formation of the yellowish BC layer that contains the bacteria, the film was washed and its impurities were eliminated. The nature of the BC was confirmed and the obtained transparent film was used for in vivo study. In the previous research, it was indicated that BC was safe and had no toxic effects in the cell culture; therefore to assess its cytotoxic effects MTT assay was not performed. *Z. officinale* root aqueous extract was prepared and its cytotoxicity effect was evaluated by MTT assay and the results revealed that it had dose-dependent toxic effects in the mouse fibroblast cell line culture. After determination of IC_{50} value of the ginger extract, it was diluted until its non toxic concentration was achieved and utilized for in vivo studies. To incorporate the herb aqueous extract to the microfibril structure of the BC, the BC film was floated overnight in the non toxic concentration of the herb aqueous extract. By this technique the herb aqueous extract was entered in the BC tiny structure.

One method for production of the bioactive wound dressing is the use of the electrospinning technique in which the polymeric nanofibers will be produced using different natural and synthetic biopolymers that some antimicrobials or antibiotics can be introduced into the electrospun film at the same time (Suwantong et al. 2008; Pilehvar-Soltanahmadi et al. 2018; Yahyaei et al. 2018). Although the mentioned procedure is acceptable, in the present study the soaking method was utilized that is a simple, safe and low-cost way of incorporation of the herb aqueous extract into the BC film.

In the present study, four different materials were used to compare their wound-healing properties with each other: honey, BC, *Z. officinale* aqueous extract, and combination of BC–*Z. officinale* aqueous extract. Assessment

of wound-healing properties of the above materials was conducted by macroscopic and microscopic examinations. The macroscopic results indicated that the BC and the herb aqueous extract had better activity than their combination in the wound contraction percentages. Hence, based on the obtained results one can conclude that this new type of bioactive wound dressing did not work properly but the microscopic examinations indicated that the combination of BC–herb aqueous extract revealed all the characteristics that each material induced in the wound site alone. For example, on day 3, the collagen bundles were observed in the group that was treated by the combination of BC–herb aqueous extract compared to the group that was treated by BC in which this phenomenon was weak. In day 7 the characteristics in the wound site in the group that was treated by the combination of BC–herb aqueous extract was similar to the BC and herb extract groups. On day 14, in the group that was treated by the combination of BC–herb aqueous extract, although the presence of stratum corneum and epidermis was similar to the two other groups, the thickness of the collagen bundles was better in the mentioned group than the other two groups. Therefore, to assess the healing efficiency of the tested materials, not only the macroscopic examinations are important but also the microscopic ones are considerable as well. Although the contraction percentage of the combination of BC–herb aqueous extract was lower than the sole effects of BC or herb aqueous extract, the microscopic examinations revealed that their combination had better effects on different wound-healing factors. So the speed of the wound healing should not be considered alone and its quality should be considered as well. In future, it is recommended to analyze the healing effects of the other traditional wound dressings in combination with the bioactive ones.

Conclusions

In conclusion, although there are different commercial products of BC, the use of the new bioactive BC products which are produced by combination of the traditional wound dressings with BC can be replaced with the previous ones. Results from this study showed that the incorporation of *Z. officinale* root aqueous extract as a traditional wound dressing to the BC film as the bioactive wound dressing had a proper effect on the microscopic factors of the wound site. *Z. officinale* root aqueous extract had dose-dependent toxic effects and it is important to analyze the toxicity of the traditional herb extracts before their in vivo usage. It is recommended to analyze the healing effects of the other traditional dressings alone and in combination with the bioactive dressings. Furthermore examination of the wounds which are treated by the new dressing products with both of the macroscopic and microscopic tests is recommended.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of the present paper.

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