



# Clinical and histopathological effects of ointment prepared from kombucha floating cellulose layer on wound healing and the activity of matrix metalloproteinase 1 in diabetic rats

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**Academic editor:** Mikhail Korokin ♦ **Received** 27 January 2022 ♦ **Accepted** 10 June 2022 ♦ **Published** 12 July 2022

**Citation:** Rajaei S, Doudi M, Setorki M, Ahadi AM (2022) Clinical and histopathological effects of ointment prepared from kombucha floating cellulose layer on wound healing and the activity of matrix metalloproteinase 1 in diabetic rats. *Research Results in Pharmacology* 8(3): 9–18. <https://doi.org/10.3897/rrpharmacology.8.81288>

## Abstract

**Introduction:** High blood glucose results in high levels of matrix metalloproteinases. Clinical and histopathological effects of the kombucha ointment on the healing of diabetic wounds were evaluated.

**Materials and methods:** This study was conducted at research Lab, Department of Microbiology, Falavarjan of Branch Islamic Azad University, Isfahan, Iran from October 2019 to September 2020. A 6 mm diameter ulcer was aseptically created on the back of forty-eight rats with streptozotocin-induced diabetes. The animals were randomly divided into 4 groups: the group that was treated with base ointment, the group that was treated with 10% kombucha ointment, the group that was treated with 20% kombucha ointment, and the group that received no ointment treatment. Then the rats in each group were divided to 4 sampling groups that were sampled on the second, fifth, tenth, and fourteenth days. Microscopic features, inflammation and vasculature and fibroblast infiltration, as well as the matrix metalloproteinase 1 (MMP1) were evaluated on days 2, 5, 10, 14 after wound healing.

**Results:** 20% kombucha ointment led to inflammation and an angiogenesis decrease compared to those in the basic group and 10%-kombucha-ointment group. Also 20% kombucha ointment led to an increase in vascular remodeling and penetration of fibroblasts. MMP1 levels increased on the second ( $p < 0.001$ ) and fifth days after wounding when treated with 10% and 20% kombucha ointment ( $p > 0.05$ ). The expression of MMP1 decreased on the ten and fourteenth days when using 20% kombucha ointment compared to the control, placebo, and 10% kombucha ointment groups ( $p > 0.05$ ).

**Discussion:** The histopathological finding indicated that both quantity and time duration of the treatment had significant effects on a degree of inflammation and angiogenesis.

**Conclusion:** Ointment prepared from 20% scoby improved the healing of diabetic ulcers within 14 days.

## Keywords

kombucha, matrix metalloproteinase, ointment.

## Introduction

Skin wounds caused by various disorders, such as burns and special diseases like diabetes, as well as severe skin damage can be life threatening (Lehmann et al. 2022). Wound healing shows an extraordinary mechanism of cellular function that is distinctive in nature (Tottoli et al. 2020). It involves the spatial and temporal synchronizations of a variety of cell types during different stages of the wound healing process, including early and inflammatory responses, as well as proliferation, migration and final phases of healing. Many people worldwide suffer from burns, bedsores or diabetic wounds (Rodrigues et al. 2018). Diabetes is a metabolic disorder (Aloulou et al. 2012; Malekaneh et al. 2015) that manifests as hyperglycemia (Aloulou et al. 2012). Various studies have reported that high blood glucose leads to abnormal persistence of the inflammatory stage, prevention of cell proliferation, elevation of matrix metalloproteinases levels, and an increase in the inflammatory cytokines (Khaksar et al. 2011). Matrix proteinases and their suppressors play key roles in the pathological changes of tissues in many diseases, including diabetes (Singh et al. 2015; Cabral-Pacheco et al. 2020). These enzymes belong to the family of metalloendopeptidases that play an important role in wound healing (Singh et al. 2015; Ayuk et al. 2016). These types of enzymes are secreted to the outer environment as zymogens or proenzymes for conducting their activation (Nikfarjam et al. 2017), for example, matrix metalloproteinase 1 is effective in epithelialising the skin, whereas matrix metalloproteinase 2 affects matrix restructuring and angiogenesis (Atyabi et al. 2018). Delay in the process of wound healing in diabetic patients has become one of the biggest challenges for physicians and health care workers. Therefore, any compound that can shorten the time of this process will accelerate the healing process (Farahpour et al. 2016). The use of herbs and herbal products for wound healing has a long history in many countries. Kombucha is a traditional drink of Asian origin (Amarasinghe et al. 2018; Jessica et al. 2018) that contains fermented black tea extract. This drink has been used to treat many health problems, including wounds in traditional medicine (Farahpour et al. 2016). Major components of kombucha tea are a floating cellulose layer (Scoby) and a sour liquid environment under it. It has an apple wine-like taste (Jessica et al. 2018). Several yeasts and bacteria co-exist with the fungi in kombucha tea. This drink contains wide ranges of amino acids, organic acids, enzymes, and vitamins (Groups B and C) and is rich in minerals. **Glucuronic acid**, which is produced in Kombucha drink, is a strong agent in the body that promotes oxidative metabolism (Moayer et al. 2017). The benefits of this extract have been based on objective observation and are less based on scientific evidence (Moayer et al. 2017). It seems that there is no accurate data based on the studies on the effect of kombucha fungi (cellulosic layer) in healing diabetic wounds until now, and no knowledge has been available pertaining to the histological study of Scoby used to treat

diabetic rat wounds. Therefore, the present study was aimed to evaluate the effect of the ointment prepared from a kombucha cellulosic layer on the healing of diabetic rat wounds based on histopathological and clinical methods and its effect on matrix metalloproteinase 1 expression in the studied rats.

## Materials and methods

### Ethical approval

The protocol of the study was approved by the Ethics Committee of Flavarjan University Branch Islamic Azad University (IR.IAU.FALA.REC.1397.036).

### Kombucha ointment preparation

In order to prepare 10% and 20% ointments, kombucha cellulose layers equal to 10 g and 20 g of its dry mass were added to 80 g and 90 g of pre-prepared base ointment, respectively. The base ointment was prepared from a combination of deionized water, paraffin, stearic acid, propylene glycol, glycerol, vaseline, cetyl alcohol, glycerolmonostearate, triethanolamine, bee wax, methyl paraben and propyl paraben (Atyabi et al. 2018).

### Animals

48 adult Wistar rats weighing (150 g to 250 g) were used in this study. The animals were held on 12 hours light/dark cycle. Water and food were available all the time, and the animals were assigned into diabetic groups. Diabetes was induced by a single intraperitoneal injection of 50 mg/kg b.w. of streptozotocin (Sigma-Aldrich, St. Louis, MO, USA). A week after the induction, a glucose level was assessed. The blood-glucose level was >300 mg/dl in all STZ injected animals (Parivar et al. 2012).

### Wounding

On the day of surgery (day zero), the rats were anesthetized with ether. The hair on the back of the body was shaved with a sterile surgical blade and disinfected. Subsequently, using a 6 mm punch, wounds with 6 mm in diameter were created between the ducts at the back of the animals. Then the rats were randomly divided into 4 groups of 12, including a control group, the treated groups that received 10% and 20% kombucha ointments, respectively, and a basic group. Each group was divided into 4 groups of 3 rats each, which were sampling groups on days 2, 5, 10, and 14 (Barati et al. 2013).

### Tissue examinations

Angiogenesis, fibroplasias, inflammatory cell infiltration were evaluated in H&E-stained sections and scored semi quantitatively as follows: no or minimal angiogenesis, fibroplasias, inflammatory cells (1); mild angiogenesis,

fibroplasias, inflammatory cells (2); inflammatory cells form extent and moderate to angiogenesis, fibroplasias (3); inflammatory cells form extent and severity to sever angiogenesis, fibroplasias (4) (Barati et al. 2013).

### Measurement of metalloproteinase 1 (MMP1) enzyme expression

On days 2, 5, 10, and 14 after wound healing, tissues were kept in normal saline for at least 1–2 hrs. Then the tissues were washed several times with normal saline to remove excess material and kept at  $-80^{\circ}\text{C}$  in normal saline with the volumes 4 times of tissue weight. For assessment of MMP1 activity, the collected tissues were removed from  $-80^{\circ}\text{C}$  and suspended for 15 minutes using a homogenizer. Finally, each tissue was centrifuged at 250 rpm for 15 minutes, and the supernatant was collected. The MMP1 levels were measured using the ELISA kit (HANGZHOU EASTBIOPHARM), according to the manufacturer proposed method (Andalibet al. 2011).

### Statistical analysis of data

The data were analyzed using the One-way analysis of variance (ANOVA) to determine the significant differences between the groups, and Dunnett's multiple comparison test as well as Tukey's post hoc test was used to compare the means. The data analyses were performed using Statistical Package for Social Sciences (SPSS) software version 16 at a P value equal to or less than 0.05.

## Results

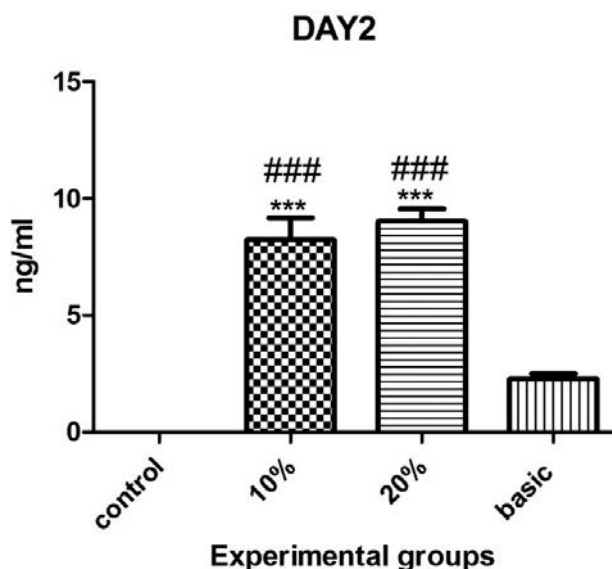
### Statistical assessment of matrix metalloproteinase 1 expression in diabetic rats

#### Matrix Metalloproteinase 1 (MMP1) expression on day 2

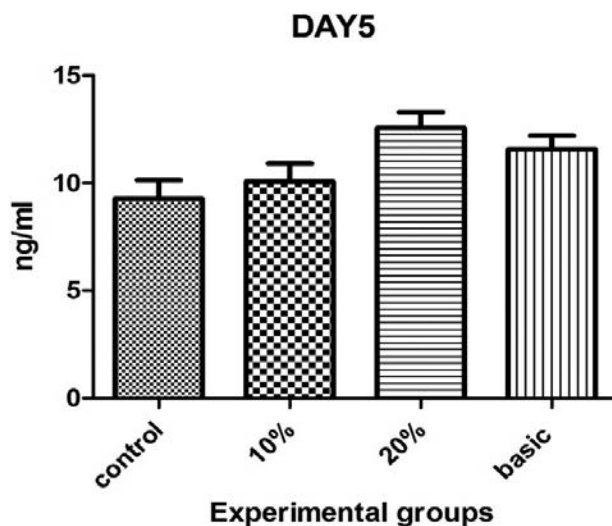
The expression levels of MMP1 were compared among the experimental groups on day 2 after wounding. The groups included: base ointment receiving diabetic group (control group), no ointment diabetic group (basic), diabetic treatment group receiving 10% kombucha ointment, and diabetic treatment group receiving 20% kombucha ointment. The expression of MMP1 was significantly increased by using 10% and 20% kombucha ointments when compared to the control and basic groups on day 2 ( $p < 0.001$ ) (Fig. 1).

#### Matrix Metalloproteinase 1 (MMP1) expression on day 5

The expression of MMP1 was increased by using 10% kombucha ointments when compared to the basic group and decreased when compared to the control group. Also, the expression of MMP1 was increased by using 20% kombucha ointments when compared to other groups, but no significant difference was observed between all the different experimental groups on day 5 ( $p > 0.05$ ) (Fig. 2).



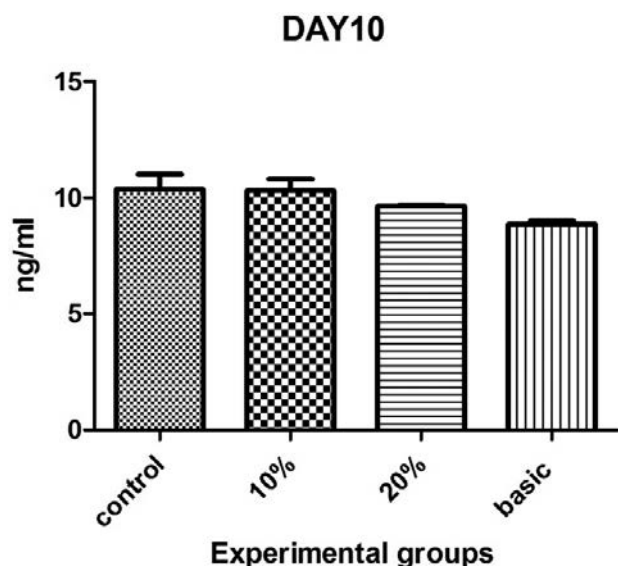
**Figure 1.** Comparison of matrix metalloproteinase (MMP1) levels between different experimental groups on the second day after wounding. The expression level of MMP1 was significantly increased by using 10% and 20% kombucha ointments when compared to control and basic groups (### and \*\*\* indicate  $p < 0.001$ ).



**Figure 2.** Comparison of matrix metalloproteinase (MMP1) levels between different experimental groups on day 5 after wounding. Also the expression of MMP1 was decreased by using 10% kombucha ointments and was increased by using 20% kombucha ointments when compared to the control group, no significant differences were seen among the experimental groups ( $p > 0.05$ ).

#### Matrix Metalloproteinase 1 (MMP1) expression on day 10

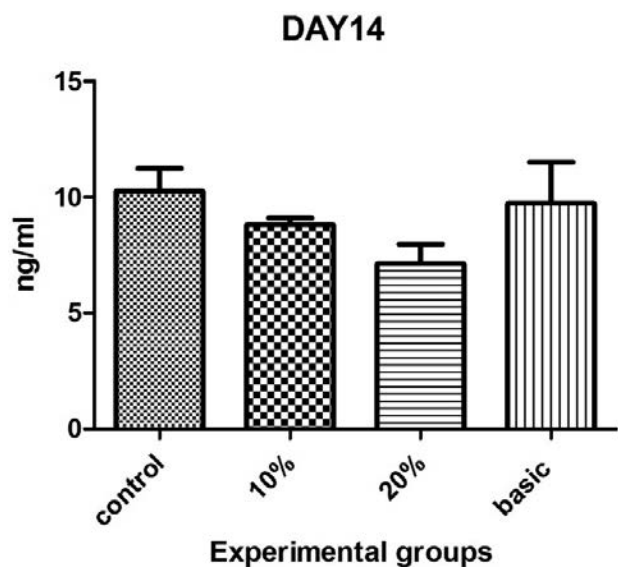
The expression of MMP1 was decreased by using 20% kombucha ointment when compared to the basic group and increased by using 10% and 20% kombucha ointments when compared to the control group, but no significant difference was observed between the different experimental groups on day 10 (Fig. 3) ( $p > 0.05$ ).



**Figure 3.** Comparison of matrix metalloproteinase (MMP1) levels between different experimental groups on day 10 after wounding. The expression of MMP1 was increased by using 10% and 20% kombucha ointments when compared to the control group, but no significant differences were seen among the experimental groups ( $p > 0.05$ ).

#### Matrix Metalloproteinase 1 (MMP1) expression on day 14

The expression levels of MMP1 were different among the experimental groups on the 14<sup>th</sup> day after wounding. There was a decrease in the expression of MMP1 by using 10% and 20% kombucha ointments when compared to the control and basic groups, but the differences between different experimental groups on the 14<sup>th</sup> day were not significant (Fig. 4) ( $p > 0.05$ ).



**Figure 4.** Comparison of matrix metalloproteinase (MMP1) levels between different experimental groups on the 14<sup>th</sup> day after wounding. The expression level of MMP1 was decreased by using kombucha ointments, but no significant differences were seen among the experimental groups ( $p > 0.05$ ).

#### Microscopic results from the use of kombucha ointment

According to Table 1, the pathological parameters indicating the progress of wound healing were reported based on scoring.

**Table 1.** Pathological parameters show the progress of wound healing and how they are ranked

Grading	Inflammation	Angiogenesis	Fibroplasia
1	absent	absent	absent
2	mild	mild	mild
3	moderate	moderate	moderate
4	severe	severe	severe

#### Macroscopic results from the use of kombucha ointment

Changes in effective parameters in skin wound healing in the study groups as well as at different times are presented in Table 2. In microscopic observations, the results of this study showed that both factor of treatment and time were highly effective on inflammation (Fig. 5). Inflammation intensity in 20% kombucha ointment treatment group, the base ointment group, and the control group. The results showed that both factors of treatment and time were effective on angiogenesis. That is, angiogenesis increased in all groups on the second day and decreased after that. Specifically, in the 20% kombucha ointment treated group, angiogenesis was not seen on the 14<sup>th</sup> day. Therefore it was concluded that angiogenesis decreased over time (Fig. 6). The results also showed that fibroblasts increased over time. So on the 14<sup>th</sup> day, in the

**Table 2.** Comparison of changes in effective parameters on skin wound healing in the studied animals at different times

Treatment group	Inflammation	Angiogenesis	Fibroplasia
A:			
2 <sup>nd</sup> day	severe	severe	absent
5 <sup>th</sup> day	moderate	moderate	mild
10 <sup>th</sup> day	mild	mild	moderate
14 <sup>th</sup> day	mild	mild	moderate
B:			
2 <sup>nd</sup> day	severe	severe	Absent
5 <sup>th</sup> day	moderate	moderate	mild
10 <sup>th</sup> day	mild	mild	moderate
14 <sup>th</sup> day	absent	absent	severe
C:			
2 <sup>nd</sup> day	severe	severe	absent
5 <sup>th</sup> day	moderate	moderate	mild
10 <sup>th</sup> day	mild	mild	moderate
14 <sup>th</sup> day	mild	mild	moderate
Z:			
2 <sup>nd</sup> day	severe	Severe	absent
5 <sup>th</sup> day	moderate	moderate	mild
10 <sup>th</sup> day	moderate	mild	mild
14 <sup>th</sup> day	mild	mild	moderate

**Note:** Group A: rats treated with 10% kombucha ointment, group B: diabetic rats treated with 20% kombucha ointment, group C: diabetic rats treated with base ointment, group Z: diabetic rats without the use of ointment.

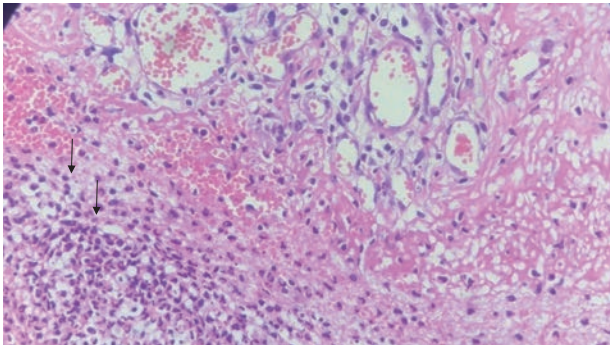


20% kombucha ointment treated group, fibroblast cell migration increased compared to that in the 10% kombucha ointment treated group, the control group and the group that had not received the ointment (Fig. 7).

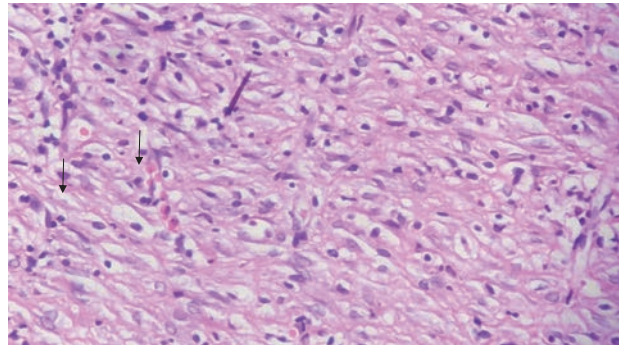
### Microscopic images of the tissue samples after kombucha ointment treatment

#### Microscopic examination of inflammation factor

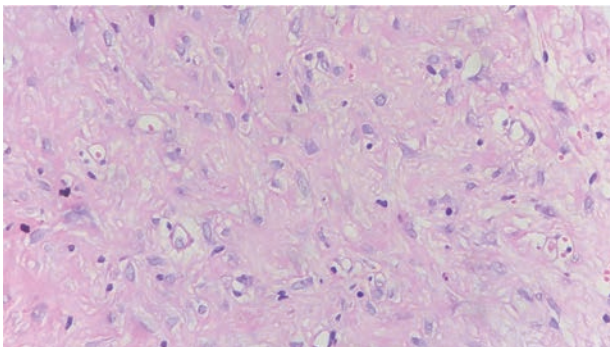
Row A



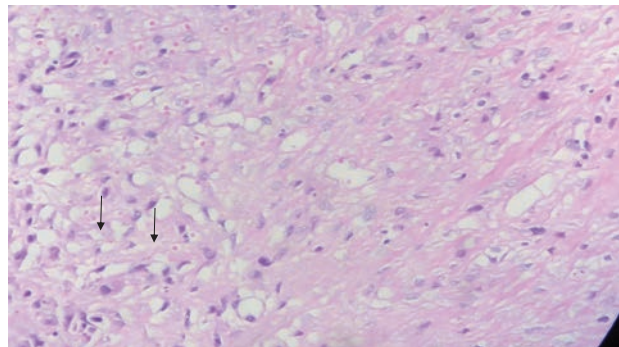
Row B: A



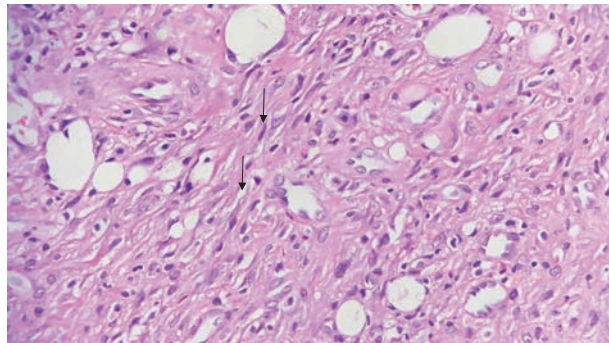
Row B: B



Row B: C



Row B: Z



**Figure 5.** Microscopic images of the skin cross section with 40× magnification. **Row A:** Investigation of the inflammatory factor on the 2<sup>nd</sup> day in all studied groups. Severe inflammation can be seen on the second day in all treatment groups, as well as in groups treated with base ointment, and without the use ointment. **Row B:** Evaluation of inflammatory factor on the 14<sup>th</sup> day of wound formation (end of treatment period). As can be seen, a small inflammation is still observed in the 10% ointment treatment group (A). There are no signs of inflammation in 20% ointment treatment group (B). In the group treated with base ointment (C), inflammation is still seen in the tissue samples. As can be seen in the group without the use of ointment (Z), inflammation and angiogenesis of blood vessels are still observed.

#### Microscopic examination of angiogenesis

See Fig. 6.

#### Microscopic examination of fibroplasia

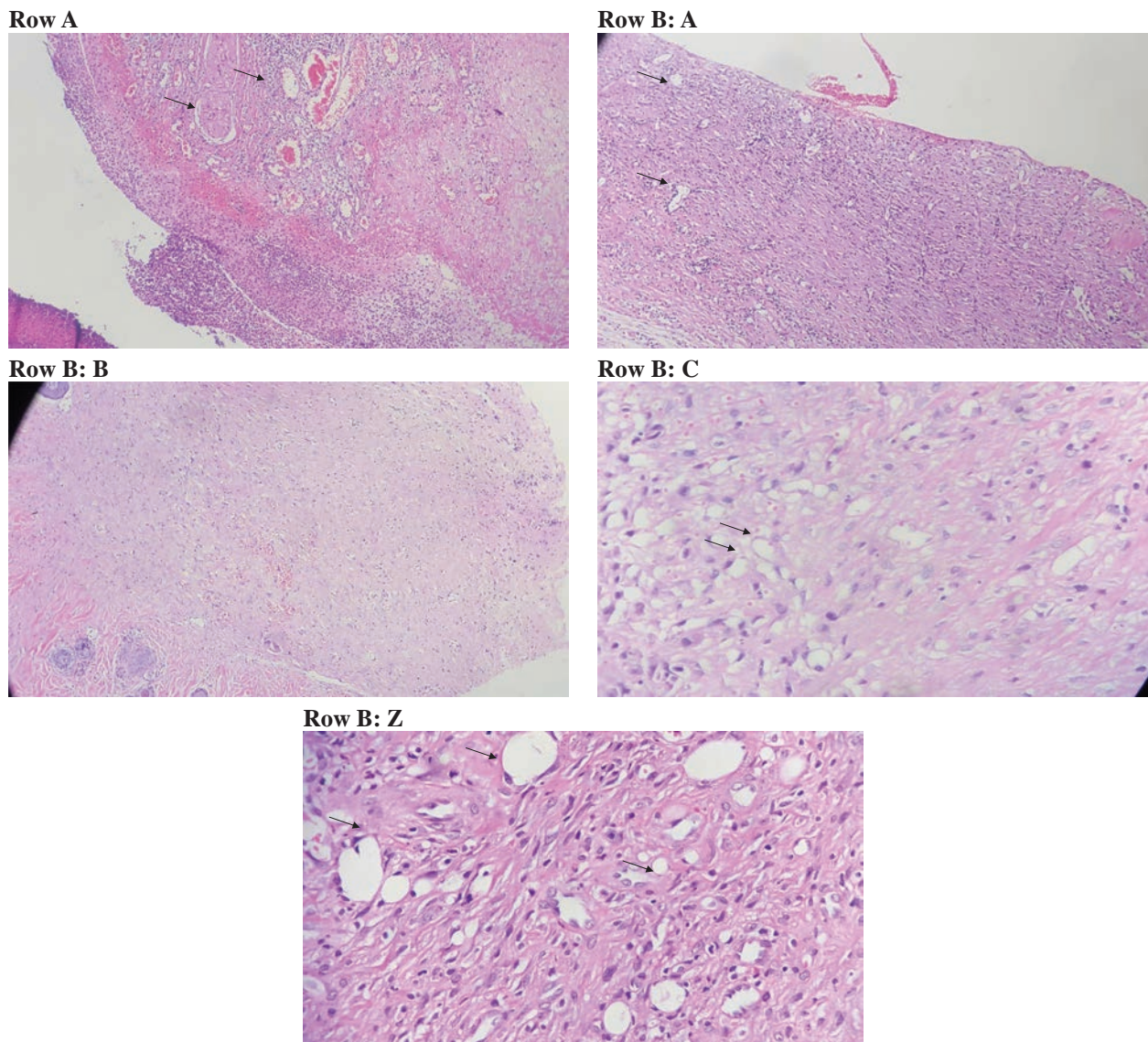
See Fig. 7.

## Discussion

Following wound occurrence, the planned healing process begins and eventually results in the cellular and molecular

status of the damaged tissue returning to its normal state. Enzymatic activity in the wound area has an important role in cellular interactions, tissue vascular remodeling as well as the formation or final arrangement of collagen fibers to restore the normal skin structure. The most important of these enzymes are from the matrix metalloproteinases family, which are classified into four groups of collagenases, gelatinases, stromelysins, and metalloenzymes based on their specific substrate and structural similarity (Atyabi et al. 2018). Matrix metalloproteinases are a group of endopeptidases that are capable of decomposing





**Figure 6.** Microscopic images of the skin cross section with 10× magnification. **Row A:** Evaluation of angiogenesis on the 2<sup>nd</sup> day in all studied groups. As can be seen, in both treatment groups (10% and 20% kombucha ointment), the base ointment group, and the group without the use of ointment, angiogenesis is observed. **Row B:** Examination of angiogenesis on the 14<sup>th</sup> day from the wound formation (end of treatment). As can be seen, small angiogenesis is still observed in the group that used 10% kombucha ointment treatment (A). In the group that used 20% kombucha ointment treatment (B), no angiogenesis is observed. In the base group (C), small angiogenesis is still observed. In the group without the use of ointment (Z), angiogenesis is clearly observed.

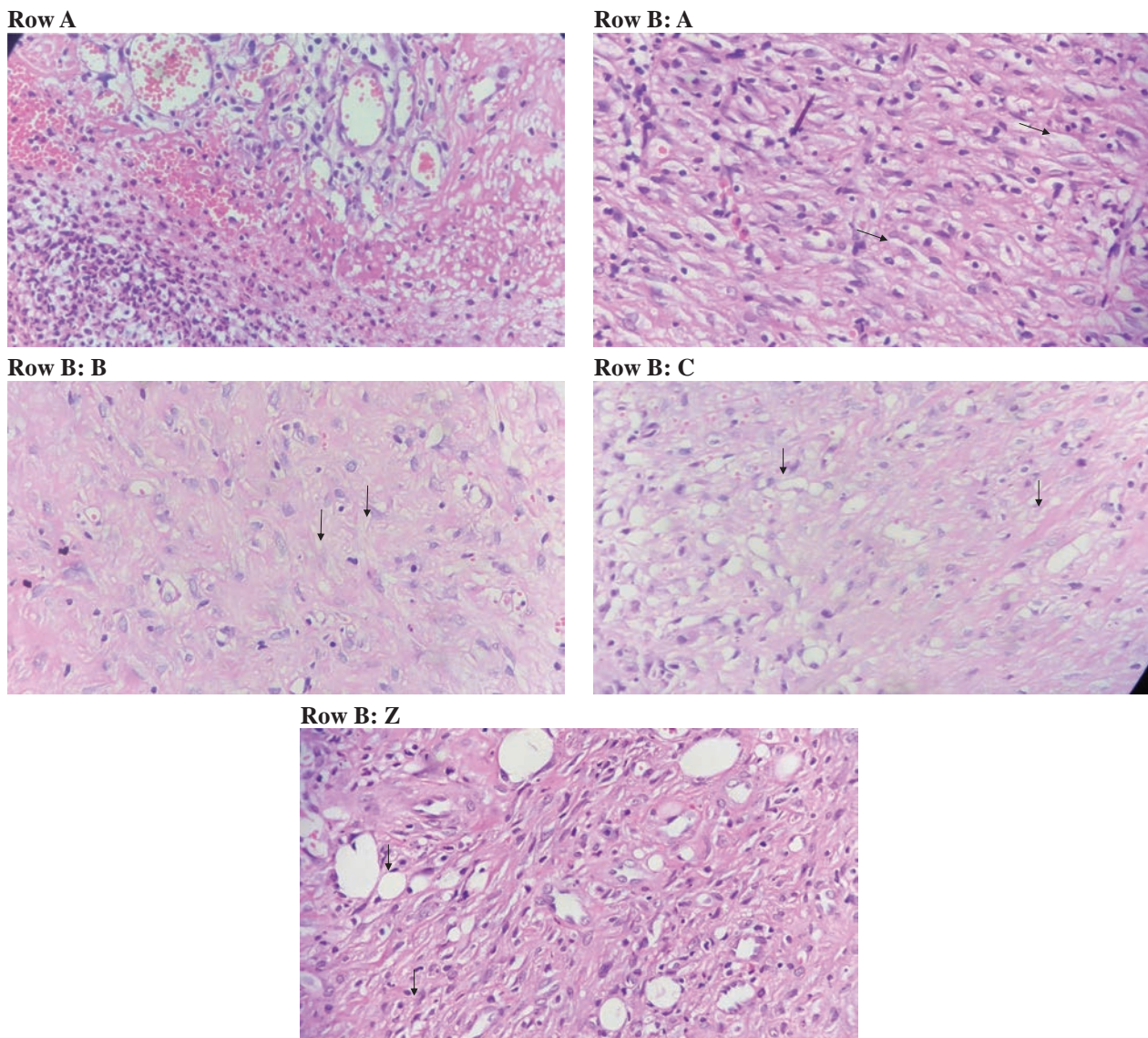
all different components of the matrix and are secreted to the activity environment as zymogen or proenzyme. Matrix metalloproteinase 2 (gelatinase) is effective in decomposing the matrix structure and angiogenesis and matrix metalloproteinase 3 affects wound healing by inducing actin network formation (Singh et al. 2015).

In the wound healing process, collagenase 1 breaks down collagen types 1 and 2, which are the major matrix of the epidermis. This enzyme, which is secreted by skin fibroblasts, is turned on or off during different stages of wound healing (Singh et al. 2015). It has been shown that MMP1 and MMP2 increase immediately after injury. Soo et al. (2000) reported that 12 hrs after an acute injury, MMP1 and MMP9 had clearly increased, with the highest levels from the first to third days, concurrent with the

tissue re-epithelialization process, and approaching the baseline after day 14 (Soo et al. 2000).

Traditional medicine has used kombucha to treat many diseases, including different kinds of wounds. Kombucha consists of a floating cellulose layer and a sour taste solution under it (Parivar et al. 2012; Mohammadi et al. 2015). In this study, the effect of ointment prepared from kombucha floating cellulose layer was studied on the healing of diabetic wounds and expression of matrix metalloproteinase 1 in male rats. This study showed for the first time that topical application of kombucha cellulose ointment accelerated the wound healing process in the rats based on macroscopic and microscopic views. Although the mechanisms involved in the healing were not detected, the beneficial advantages of kombucha cellulose layer by one of





**Figure 7.** Microscopic images of the skin cross section with 10× magnification. **Row A:** Evaluation of fibroplasia on the 2<sup>nd</sup> day in all studied groups. As can be seen, in both treatment groups (10% and 20% kombucha ointment), the base ointment group, and the group without the use of ointment, no fibroblast cells are observed. **Row B:** Examination of fibroplasia on the 14<sup>th</sup> day from the wound formation (end of treatment). As can be seen, fibroblasts are observed in the group that used 10% kombucha ointment treatment (A). In the group that used 20% kombucha ointment treatment (B), the presence of fibroblasts on the tissue surface is quite evident. In the base group (C), slight fibroblasts are observed. In the group without the use of ointment (Z), fibroblasts are not seen. Comparing the above results, it was shown that the function of 10% kombucha drug, the control group and the base ointment treatment group were the same, but by using 20% kombucha treatment, an increase in spindle-shaped cells and fibroblasts was observed.

the most important bacteria (*Acetobacter xylinum*) in the fungal tissue was cited in previous studies. The kombucha floating cellulose layer is mainly made of cellulose polymer (Barati et al. 2013). Cellulose is the most abundant polymer in nature and consists of glucose units with beta-glycoside bonds. This polymer is extremely lightweight and does not dissolve in most aqueous solvents (Mondal 2016). Bacterial cellulose seeps out of the gaps in the cell wall. Because of low density, the cellulose strands move to the upper part of the kombucha fluid upon extraction and join together at the liquid surface to form the cellulose disc. This floating disk in addition to polysaccharides contains some fat composed of sphingolipids (Mondal 2016).

About 1 to 2% of the sugars in kombucha are consumed by the yeast to form sphingolipid content of the surface disk. The microorganisms that exist in kombucha adhere to this disk and form microbial masses called colony (Jessica et al. 2018). Bacterial cellulose is pure and consists of about 99.8% water and 0.2% polysaccharide. The arranged and delicate structure of the bacterial cellulose in kombucha, as well as its tensile strength, high water-holding capacity, ability to exchange water vapor with the environment, nano-sized pores for molecules entry, flexibility, high biodegradability, tissue biocompatibility, non-allergenicity, and highly adapted to the immune system has resulted in receiving special attention to it in biomedicine and other

industries. In addition, the morphological similarity of bacterial cellulose to cellular collagen made it a candidate for cell tolerance and support (Gorgieva and Trcek 2019). Due to the small size of bacterial cellulose fibers, this product is widely used in medicinal procedures, such as wound dressing, as well as artificial skin, and blood vessel construction (Lahiri et al. 2021). Recent studies have been conducted on the usage of bacterial cellulose as blood vessels (Charpentier et al. 2006). In addition, the non-dried cellulose membrane is a high nanoporous material that is capable of passing antibiotics or other drugs to the wound although creates an effective physical barrier against any external infections (Cai et al. 2010). Also due to high mechanical strength of bacterial cellulose and its permeability for liquids and gases in wet conditions, pure and wet cellulose can be used for pain reduction in damaged skin and for wound healing (Lahiri et al. 2021), as it is used to cover a variety of wounds (Czaja et al. 2006). The bacterial wet cellulose has a clear perspective for usage as artificial skin in the treatment of severe burn wounds and blistering of skin due to various chemical and physical factors that are present in it (Cai et al. 2010). In tissue engineering, a three-dimensional extracellular matrix is commonly employed to act as a scaffold keeping cells in a constant state to control tissue structure content and regulate the behavior of cells. Bacterial cellulose, which consists of bio-compatible nanofibers, has been suggested as a potential scaffold in the production of dental implants, vascular grafts, catheter coatings, coatings for cardiovascular stents, cranial stents and controlled drug delivery carriers (Gorgieva and Trcek 2019). It is also claimed that topical application of kombucha has a good effect on the vitality and freshness of the skin. Therefore, this cellulose can be used to heal burns and other skin injuries, perhaps due to the presence of live yeasts, which have an effective role in the rejuvenation and healing of the wound (Ayuket al. 2016).

In addition to cellulose, *Acetobacter xylinum* is capable of producing other polysaccharides, such as N-acetylglucosamine containing polymers. These polysaccharides are decomposed by lysozyme in body fluids, producing N-acetyl glucosamine that accelerates the healing process (Lahiri et al. 2021). In addition, N-acetyl glucosamine, along with **glucuronic acid**, produce one of the **hyaluronic acid** - forming disaccharides found in the cartilage gland tissue. In this regard, Mghsoudi et al. (2009) showed the effect of kombucha in removing intraperitoneal adhesions after surgery and attributed it to oxidized restored cellulose and **hyaluronic acid** membranes (Mghsoudi et al. 2009).

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- Lee et al. (1995) showed that **hyaluronic acid** stimulates fibroblast proliferation and initiates epithelialization (Lee et al. 1995), which is consistent with the findings of this study by using kombucha containing ointment. It is mentioned that fibroblasts play an important role in the production and secretion of matrix metalloproteinase in addition to wound healing (Parivar et al 2012). studied the effect of kombucha solution on wound healing in male mice. They studied the effect of kombucha solution on wound healing in male mice. They showed that topical usage of the solution led to a significant acceleration in the wound healing process and attributed this property to the presence of **glucuronic acid** in the solution (Parivar et al. 2012). Also according to the findings by Longaker et al. (1991), the amount of **hyaluronic acid** is reduced 5–10 days after injury, and therefore kombucha colonies can be used to increase this vital metabolite in the process of wound healing (Longaker et al. 1991). Pasonen et al. (2003) reported that external hyaluronic administration induces the proliferation of keratinocytes, which are required for epithelial remodeling (Pasonen-Seppanen et al. 2003). More studies are required on the effects of many mentioned ingredients of kombucha, such as vitamins, antibiotics, and organic acids, which are produced by yeasts and bacteria in addition to the role of the bacterial colonies attached to the underside of the floating cellulose layer on the healing process of wounds, especially diabetes wounds.

## Conclusion

According to the results of this study, it is concluded that ointment prepared from kombucha cellulose floating disk was able to improve the healing process of diabetes wounds, as well as the induction of matrix metalloproteinases expression, which are some of the effective factors in wound healing histopathological process. Therefore, kombucha nanocellulose layer is proposed for the production in scaffold areas, such as the production of dental implants, vascular grafts, catheter coatings, cardiovascular and cranial stents coatings, and controlled drug delivery carriers. Also the ointment prepared from kombucha floating cellulose layer) scoby( is proposed for manufacturing medicinal dressings.

## Conflict of interests

The authors declare no conflict of interests.



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