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KAEMPFEROL PROMOTES WOUND-HEALING IN DIABETIC RATS THROUGH ANTIBACTERIAL AND ANTIOXIDANT EFFECTS, DEVOID OF PROLIFERATIVE ACTION

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Abstract

The investigation of novel phytochemicals for the prevention and treatment of infections caused by multidrug-resistant pathogens is gaining attention. The current study evaluated the *in-vitro* antimicrobial activity of kaempferol against methicillin-resistant *Staphylococcus aureus (*MRSA) and *Pseudomonas aeruginosa.* Its *in-vivo* efficacy in inhibiting these pathogens was determined using an excision wound model in nicotinamide-streptozocin- -induced diabetic rats. Kaempferol displayed an inhibitory effect against the tested bacteria both *in vitro* and *in vivo*. It also healed excision wounds at a 1% (*w*/*w*) concentration. An increase in antioxidant enzymes in wounded tissue was observed on kaempferol treatment. A reduction in the MRSA and *P. aeruginosa* counts in wounded tissue together with a reduced epithelization period was observed when compared to the infected control. A thicker epithelium, new capillaries, and a decrease in inflammatory cells were detected by hematoxylin and eosin staining. Furthermore, an increase in collagen fibers and their deposition was observed by Masson's trichrome staining. Kaempferol at 40 µM did not display any toxicity for human keratinocytes grown in media containing high glucose and it did not affect the expression of the pro-healing cytokines genes vascular endothelial growth factor (VEGF) and transforming growth factor-β-1 (TGFβ1). Kaempferol displayed antibacterial and antioxidant actions but did not increase the expression of proliferative genes.

Keywords: Epithelization. Excision wound. TGF-β1. VEGF.

1. Introduction

Chronic wounds, particularly non-healing, persistent diabetic wounds, are a major global health problem. Wounds in diabetic individuals require special attention and longer treatment compared to those of non-diabetic patients. The restoration of skin integrity fails and becomes unbalanced, resulting in pathological complications, leading to pain, morbidity, and high-cost treatments for diabetes (Feng et al. 2022). While new synthetic molecules are being developed in modern medicine, a large number of natural products are gaining attention due to their proven effects in folk medicine (Liu et al. 2022).

Bioactive components from plants have been shown to improve wound contraction and epithelization in different phases of wound healing. In diabetic conditions, a substance with antioxidant and antimicrobial activities may promote molecular, cellular, and tissue restoration (Wang et al. 2021). Additionally, an infection of diabetic wounds exacerbates skin wounds and decreases the effectiveness of

antimicrobial agents, leading to recurrent infection and treatment failures (Rodríguez-Rodríguez et al. 2022).

Flavonoids are phenolic compounds naturally synthesized in plants and have a range of pharmacological effects that include antidiabetic, antimicrobial, neuroprotective, cardioprotective, and antioxidant (Panche et al. 2016). Kaempferol is a polyphenolic compound found in many plants, including several vegetables (Ren et al. 2019). Kaempferol and plant extracts containing kaempferol have been reported to display wound-healing activity *in vivo* in non-infected animals (Ambiga et al. 2007; Özay et al. 2019; Hu et al. 2020; Soib et al. 2020). Furthermore, the effect of kaempferol in the healing of pathogen-infected wounds has not, to our knowledge, been investigated.

The current study provides insights into the effect of kaempferol in healing infected diabetic wounds and its probable mechanism of action. Agents that elevate growth factor expression and scavenge oxygen free radicals are known to promote wound healing (Xu et al. 2020; Vaidyanathan 2021). One of the growth factors that promote healing via angiogenesis is vascular endothelial growth factor (VEGF). Another growth factor involved is transforming growth factor-beta1 (TGFβ1), which is known to play a role in wound healing and scar formation (Penn et al. 2012). Reactive oxidative species (ROS) are implicated in delayed wound healing, and these are known to reduce the levels of antioxidant enzymes, including superoxide dismutase (SOD) and catalase in wounded tissue (Rasik and Shukla 2000). Elevated antioxidant enzyme levels induced by different agents in the wounded tissue is an indication of scavenging of the reactive oxygen species (Comino-Sanz et al. 2021).

This is the first and a novel study showing that flavonoids have antimicrobial effects on infected wounds. Our recent studies on *Moringa oleifera* methanol extract showed its wound-healing effect in infected diabetic wounds (Al-Ghanayem et al. 2022). Because *M. oleifera* contains several constituents, it is difficult to investigate the effect of all the constituents together in one study. Kaempferol is one of the polyphenols present in moringa as well as many other plants. Therefore, it was selected first to determine the wound-healing action. On the basis of the earlier studies on moringa, two of the most common bacterial pathogens responsible for wound infections, methicillin-resistant *Staphylococcus aureus* (MRSA) (Gram-positive cocci) and *Pseudomonas aeruginosa* (Gram-negative bacilli), were used to infect wounds. To investigate the chemical constituent(s) of *M. oleifera* responsible for wound-healing activity in diabetic wounds, the current study determined the wound-healing property of kaempferol in infected excision wounds in diabetic rats. The inhibitory action of kaempferol on the pathogens and its antioxidant activity were evaluated *in vivo*. To further investigate its mechanism, an *in-vitro* study on human keratinocyte (HaCaT) cells was undertaken to evaluate its effect on VEGF and TGFβ1 gene expression.

2. Material and Methods

Materials

Kaempferol was purchased from MedChemExpress (# HY-14590, New Jersey, United States). The chemicals used were all of analytical grade. MRSA (ATCC 43300) and *P. aeruginosa* (ATCC 27853) were used.

Preparation of kaempferol ointment

Kaempferol is a yellow crystalline powder having a bitter taste, with slight solubility in water and good solubility in ethyl alcohol and other solvents such as diethyl ether. The ointment base was formulated by mixing glycol stearate, 1,2-propylene glycol, and paraffin oil at a ratio of 3:6:1. Kaempferol was added to the base preparation to obtain 0.5% kaempferol ointment (*w*/*w*) and 1% kaempferol (*w*/*w*). The base was selected based on earlier reports (Özay et al. 2019). The prepared ointment was tested for color and odor, homogeneity, washability, loss on drying, pH, and spreadability using protocols outlined elsewhere (Nayeem et al. 2021). The diffusion ability was tested in agar medium (Jun and Bayoumi 1986). Stability was tested at different temperatures for 3 months (Nayeem et al. 2008).

Antimicrobial activity

Antibacterial activity was determined by a well-diffusion assay. Mullen Hilton broth was employed to obtain a minimum inhibitory concentration (MIC). To determine the minimum bactericidal concentration (MBC), mannitol salt agar was utilized for MRSA and cetrimide agar media was used for *P. aeruginosa* (Ekom et al. 2022).

Animals

Inbred male rats (Wistar strain) aged 4–4.5 months and weighing 210–230 g, were used. Because pathogenic bacteria were used, personnel involved in the experiment exercised maximum precaution to avoid the transmission of pathogens and experimental animals were kept in isolation. The research methodology was approved by the Shaqra university regulatory body (Ethical Research Committee, number ERC_SU_20220091).

Skin irritation test

An area of 500 mm^2 was depilated on the back of rats and kaempferol ointment was applied. Signs of irritation or inflammation were monitored at 12 h intervals for 72 h (Ankomah et al. 2022).

Nicotinamide-streptozocin-induced diabetes

Overnight-fasted rats were administered nicotinamide (120 mg/kg) in citrate buffer by an intraperitoneal route. After 15 min, streptozocin (60 mg/kg) was given by intraperitoneal injection. The streptozocin was prepared in citrate buffer and the volume of injection did not exceed 1 ml/kg body weight. Serum glucose levels (fasting) were determined after 72 h and animals were considered to be diabetic if the serum glucose level was ≥150 mg/dl (Al-Ghanayem et al. 2022).

Wound-healing activity

The rats were anesthetized using an anesthetic cocktail consisting of ketamine (90 mg/kg) and xylazine (9 mg/kg) by an intraperitoneal route at a dose of 1 mL/kg (Sotoudeh et al. 2022). An area of 500 mm^2 on the back of the animal was depilated and skin was cut off to create an excision wound. Bacterial culture (30 µL) containing 10^8 colony forming units per ml (CFU/mL) was introduced into the wounded area (Al-Ghanayem et al. 2022). Diabetic animals were divided into groups of twelve animals each and four groups were used for each pathogen as follows: group I was the control (ointment base); group II and group III were treated with kaempferol ointment at 0.5% *w*/*w* and 1%*w*/*w* concentrations, respectively; and the final group was treated with an antibiotic (2% mupirocin for MRSA and0.1% gentamicin for *P. aeruginosa*). Of these 12 rats, 6 animals were used for the determination of wound contraction every fourth day till day 24. The wound area was measured by tracing the wound on a transparent sheet and a graph paper was superimposed on the traced area to determine the total wounded area. On day 24, animals were euthanized using 5 times of the anesthetic dose of ketamine and xylazine and the skin tissue was used to determine catalase (Link 1988) and (SOD) activities (Elstner and Heupel 1976) and microbial load (CFU/g tissues) and for histological examination (Al-Ghanayem et al. 2022). Hematoxylin and eosin (H&E) or Masson's trichrome stain were used to evaluate the histological changes. The epithelization period, which is the complete healing of wounds, was determined in the remaining six animals.

Cytotoxic assay and VEGF and TGF-β1 gene expression of HaCaT cells

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and study on VEGF and TGF-β1 gene expression were performed as described elsewhere (Al-Ghanayem et al. 2022). Kaempferol at different concentrations (0–100 µM) was added to the HaCaT cells to determine its toxicity.

Kaempferol at nontoxic concentrations (20 µM and 40 µM) was used to study its effect on gene expression. The primers used for DNA amplification are given in Table 1.

Table 1. Primer sequences for the gene expression study.

Gene	Forward	Reverse
GAPDH	5'CGGAGTCAACGGATTTGGTCGTAT3'	5'AGTCTTCTCCATGGTGGTGAAGAC3'
TGFβ1	5'CTTCTCCACCAACTACTGCTTC3'	5'GGGTCCCAGGCAGAAGTT3'
VEGF	5'CTGGCCTGCAGACATCAAAGTGAG3'	5'CTTCCCGTTCTCAGCTCCACAAAC3'

Statistical analysis

Results are mean ± standard error of mean (SEM). Analysis of variance (one-way) with a post-test (Tukey's) was used to assess statistical significance (SPSS version 20 for Windows). The *p* values are represented by asterisks as **p* <0.5, ***p* <0.01, and ****p* <0.001.

3. Results

Kaempferol ointment displayed good stability and had a bitter in taste when kept in the mouth. The diffusion ability, homogeneity, spreading ability, and washability were determined and found to be satisfactory (Table 2). The ointment preparation did not produce any signs of irritation or inflammation when applied to intact skin.

Table 2. Physicochemical properties of kaempferol ointment.

Parameter	Result
Color	Slightly yellowish
Odor	Odorless
Taste	Bitter
Spreadability (seconds)	12 s
Diffusion	0.7 cm
Stability	Stable at 40 °C, 24 °C and 37 °C
Washability	Satisfactory
Homogeneity	Good

***In-vitro* antibacterial activity**

The Kaempferol formulation displayed inhibitory effects against MRSA (MIC-256 µg/mL) and *P. aeruginosa* (MIC-512 µg/mL). However, the effect was greater against MRSA when compared to *P. aeruginosa* (Table 3).

Table 3. Inhibitory effect of kaempferol (µg/mL) against the tested bacterial pathogens.

Bacteria	Kaempferol (µg/mL)	
	MIC	MBC
P. aeruginosa	512	1024
MRSA	256	512

Wound-healing effect in an MRSA-infected excision wound in diabetic rats

Nicotinamide-streptozocin-treated animals exhibited signs of diabetes such as polyuria, polydipsia, and polyphagia. Animals lost weight but without mortality because animals with a very high blood glucose level of >250 mg/dl were excluded from the study.

Wounds treated with mupirocin (2%) had a better effect on epithelization than the higher kaempferol concentration (1% *w*/*w*) (*p* <0.001). No significant difference was observed with the low-concentration kaempferol ointment (0.5% *w*/*w*) compared to the base-treated control (*p* <0.001) (Figure 1). The effect on wound contraction showed that wound closure was the most rapid in antibiotic-treated

wounds. The higher kaempferol concentration (1% *w*/*w*) was less effective compared to the antibiotic, although the difference between them was not significant. The lower kaempferol-concentration (0.5% *w*/*w*) ointment had the least effect on wound contraction, confirming the dose-dependent effect of kaempferol. A significant effect of the low kaempferol dose was observed after 12 days (p <0.001), and its effect was less significant on days 20 (p <0.05) and 24 (p <0.01) when compared to the control (Figure 2).

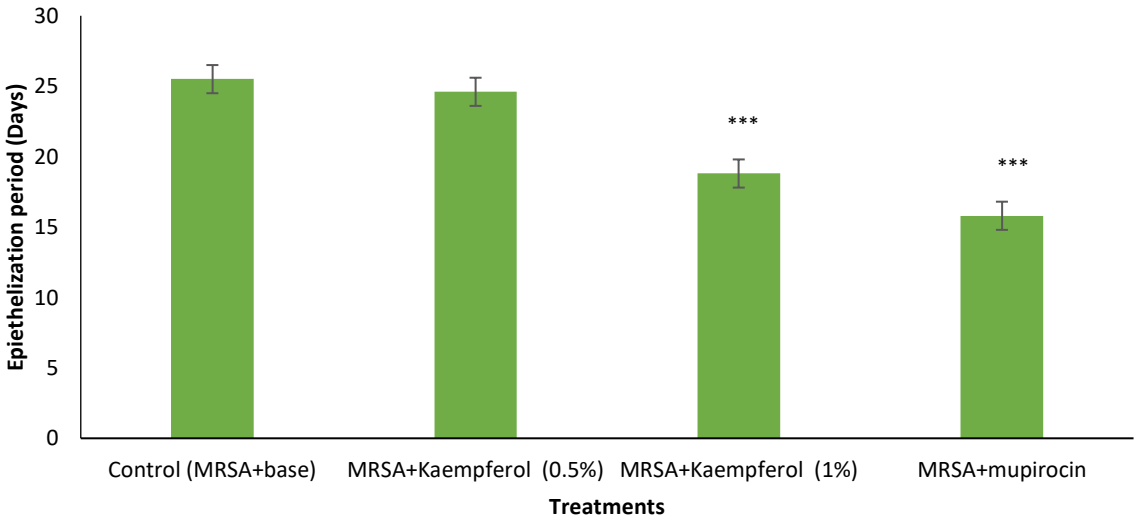

Figure 1. Duration of epithelization in animals infected with MRSA (positive control-mupirocin 2%). Bars indicate mean ± SEM, n = 6, ***p <0.001, compared to the base-treated control.

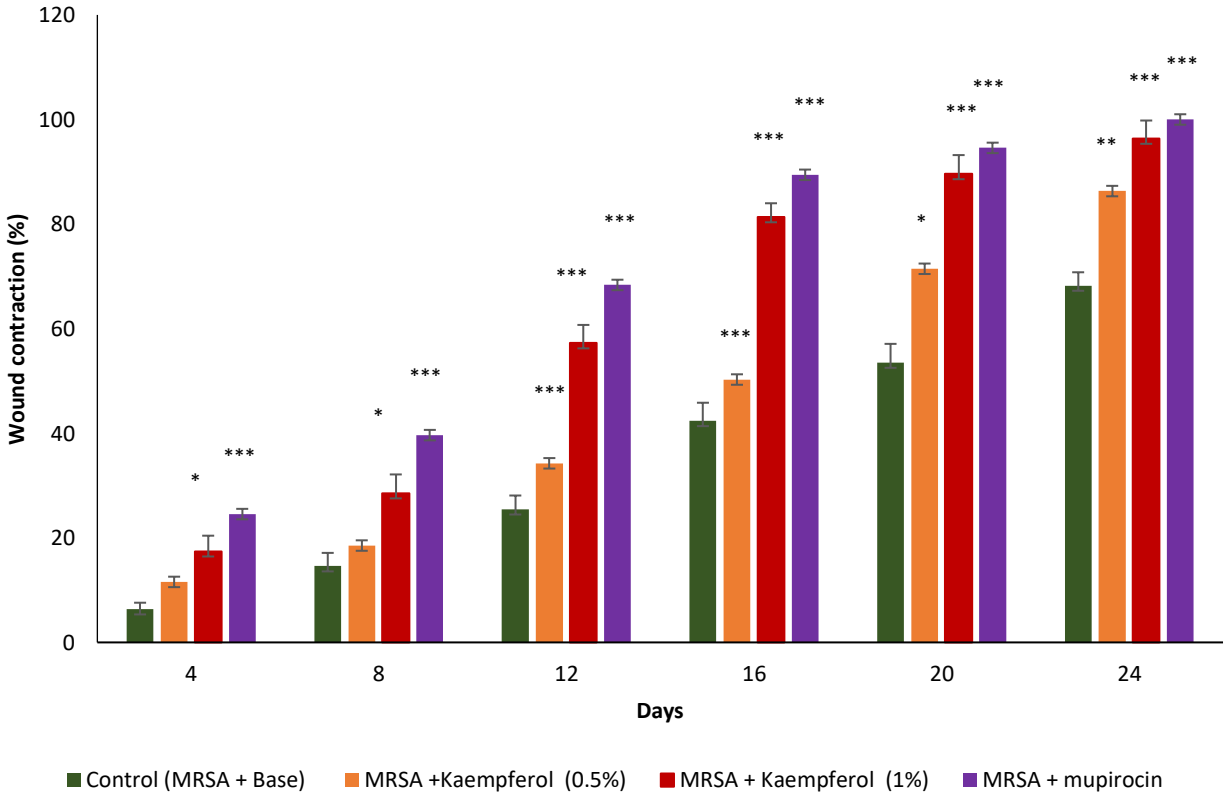

Figure 2. Wound contraction (%) after MRSA infection (positive control—mupirocin 2%). Bars indicate mean ± SEM, n = 6, *p <0.05, **p <0.01, and ***p <0.001, compared to the base-treated control.

Histological examination supported the macroscopic observation. In the base-treated animals, epithelial regeneration was incomplete, with the presence of inflammatory cells and poor angiogenesis. Mupirocin- and kaempferol (1%)-treated animals exhibited complete epithelial regeneration with a lesser number of inflammatory cells, while there were few inflammatory cells in the kaempferol (0.5%)-treated animals (Figure 3a). The most limited wound-healing effect was observed with the lower kaempferol concentration (0.5% *w*/*w*). The effect on collagen deposition was similar to the effects observed on epithelization. Collagen deposition was least in the base-treated group and maximum collagen deposition was observed after mupirocin application (Figure 3b).

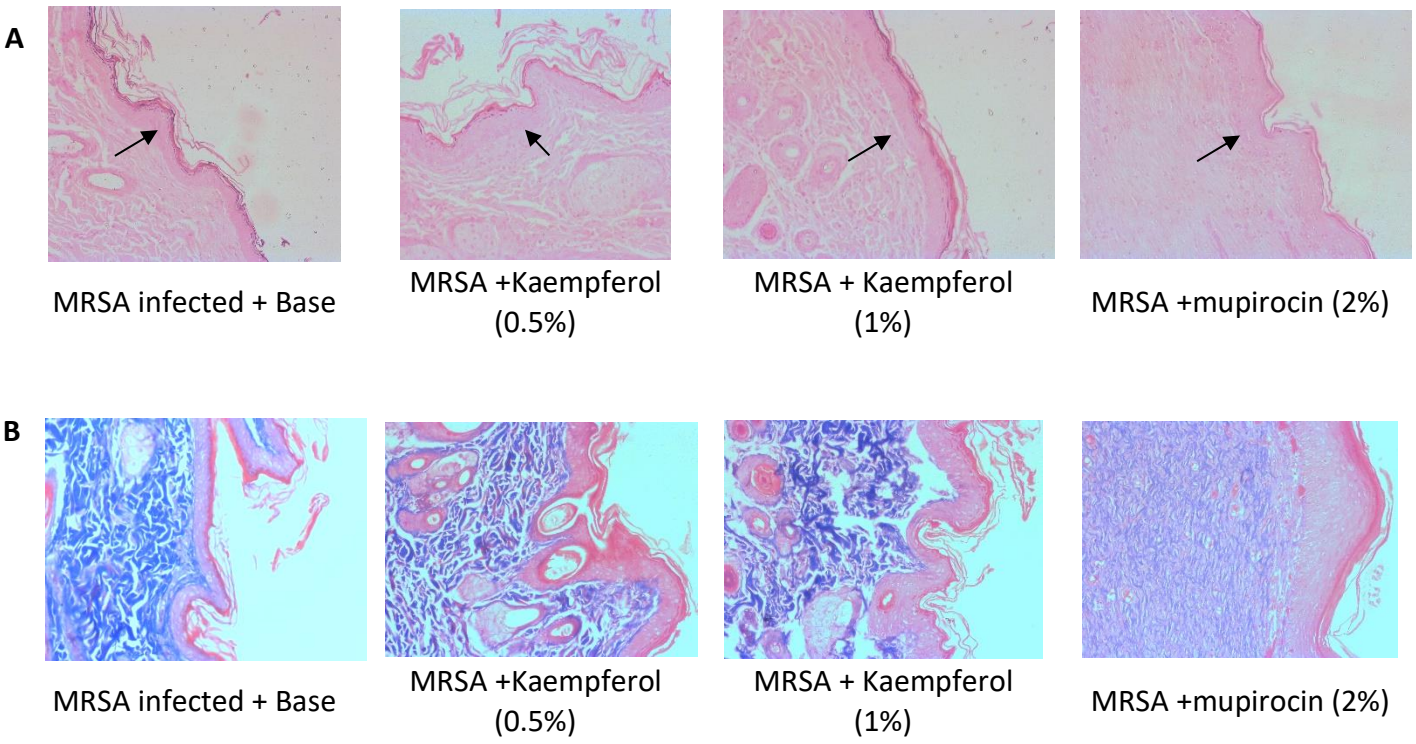

Figure 3. A - Histology of skin after MRSA infection (H&E staining) showing epidermis (black arrow), angiogenesis, and inflammatory cells (200×). B - Histology of skin (Masson's trichrome stain) after MRSA infection showing collagen deposition (blue color) (200×).

The enzyme activities in the wounded tissues were evaluated on day 24. The SOD and catalase activities were significantly increased in the groups treated with either kaempferol concentration when compared to the base-treated control (p <0.001). However, the enzyme activities were relatively less after mupirocin treatment (p <0.001) (Figure 4).

The bacterial load on day 24 showed that the lower kaempferol concentration did not significantly decrease the bacterial load compared to the control, whereas the higher concentration was moderately effective (p <0.05). As expected, mupirocin decreased the bacterial load extensively compared to the control (p <0.01) (Table 4).

Table 4. MRSA count in wounded tissue.

Group	Log_{10} CFU/g of Tissue
Control (base)	9.47 ± 1.24
Kaempferol (0.5% *w*/*w*)	5.71 ± 1.89
Kaempferol (1% *w*/*w*)	3.25 ± 1.43 *
Mupirocin (2%)	1.30 ± 1.10 **

Values shown are mean ± SEM, n = 6, * p<0.05, **p<0.01 in comparison to the control (base).

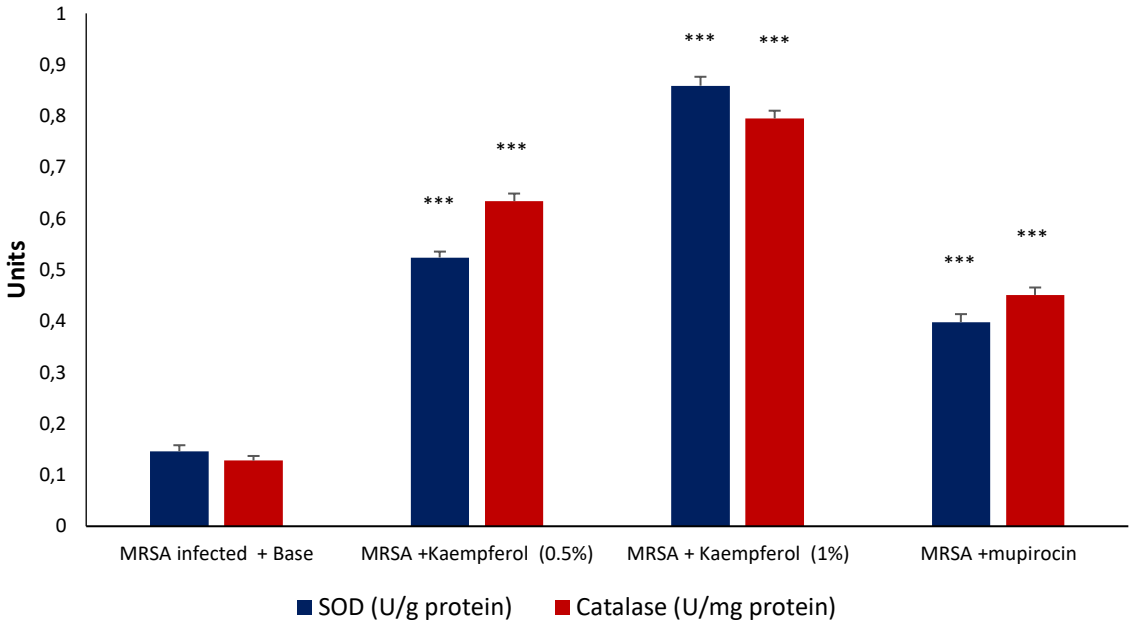

Figure 4. Antioxidant enzyme activities after MRSA infection in the wounded tissues. Bars indicate mean ± SEM, n = 6, ***p <0.001, compared to the base-treated control.

Wound-healing effect after *P. aeruginosa* infection

Severe wound infection was observed in all groups of animals with mortality (approximately 30% in the control group). More animals were added to obtain the required sample size in the control group. The epithelization period was longer in the infected control group and the lower kaempferol concentration (0.5% *w*/*w*)-treated group. In contrast, the application of higher kaempferol (1% *w*/*w*) accelerated skin epithelium regeneration to within 19–20 days (Figure 5). Gentamicin (0.1%) application had the greatest effect among all the treatments, as indicated by a short epithelization period. The measurement of wound size every fourth day showed that the lower kaempferol concentration (0.5% *w*/*w*) was ineffective in improving wound contraction when compared to the base-treated control. The higher kaempferol concentration (1% *w*/*w*) was less effective against *P. aeruginosa* when compared to MRSA. The antibiotic-treated group displayed an extremely significant wound-healing effect from the fourth day onward (Figure 6).

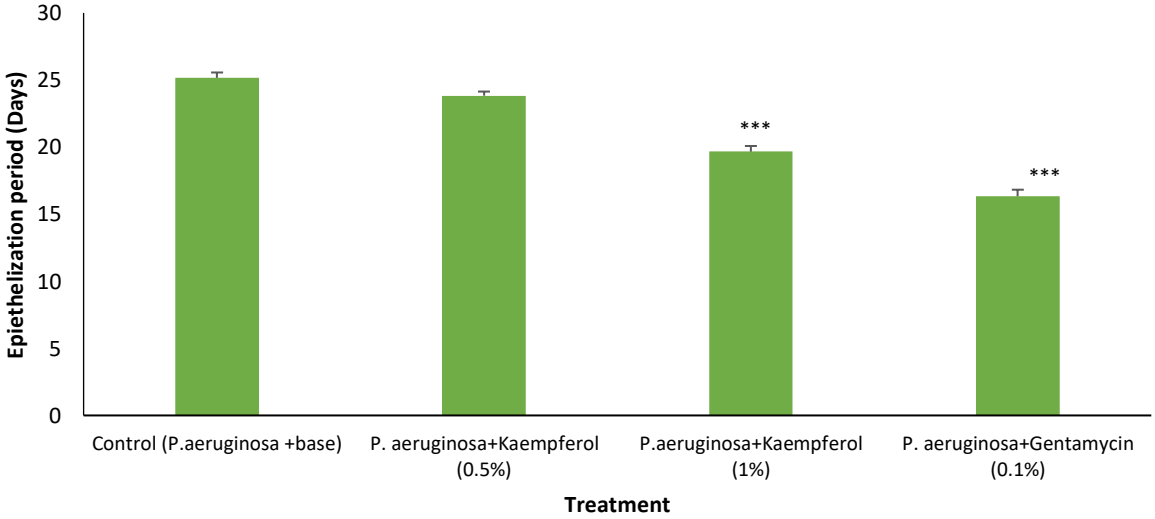

Figure 5. Period of epithelization in animals infected with *P. aeruginosa*. Bars indicate mean ± SEM, n = 6, ***p <0.001, compared to the base-treated control (base).

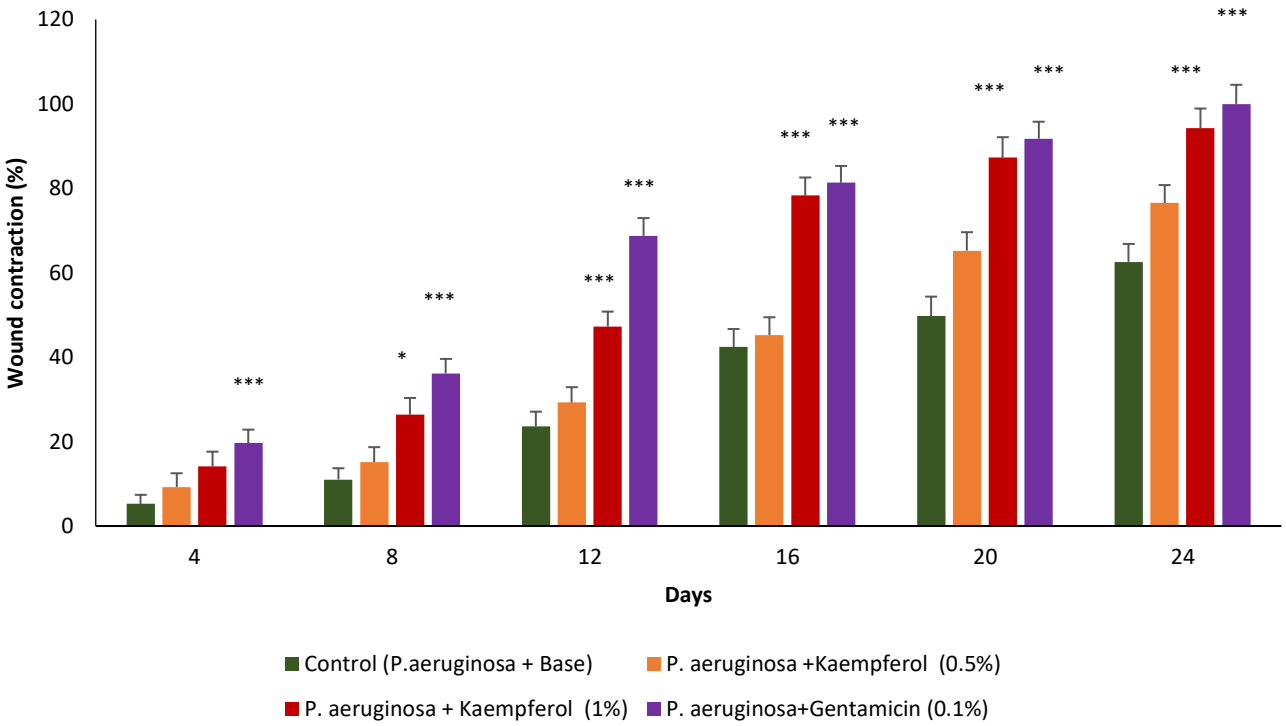

Figure 6. Wound contraction (%) after *P. aeruginosa* infection. Bars indicate the mean ± SEM, n = 6, *p<0.05, **p<0.01, ***p<0.001, compared to the base-treated control.

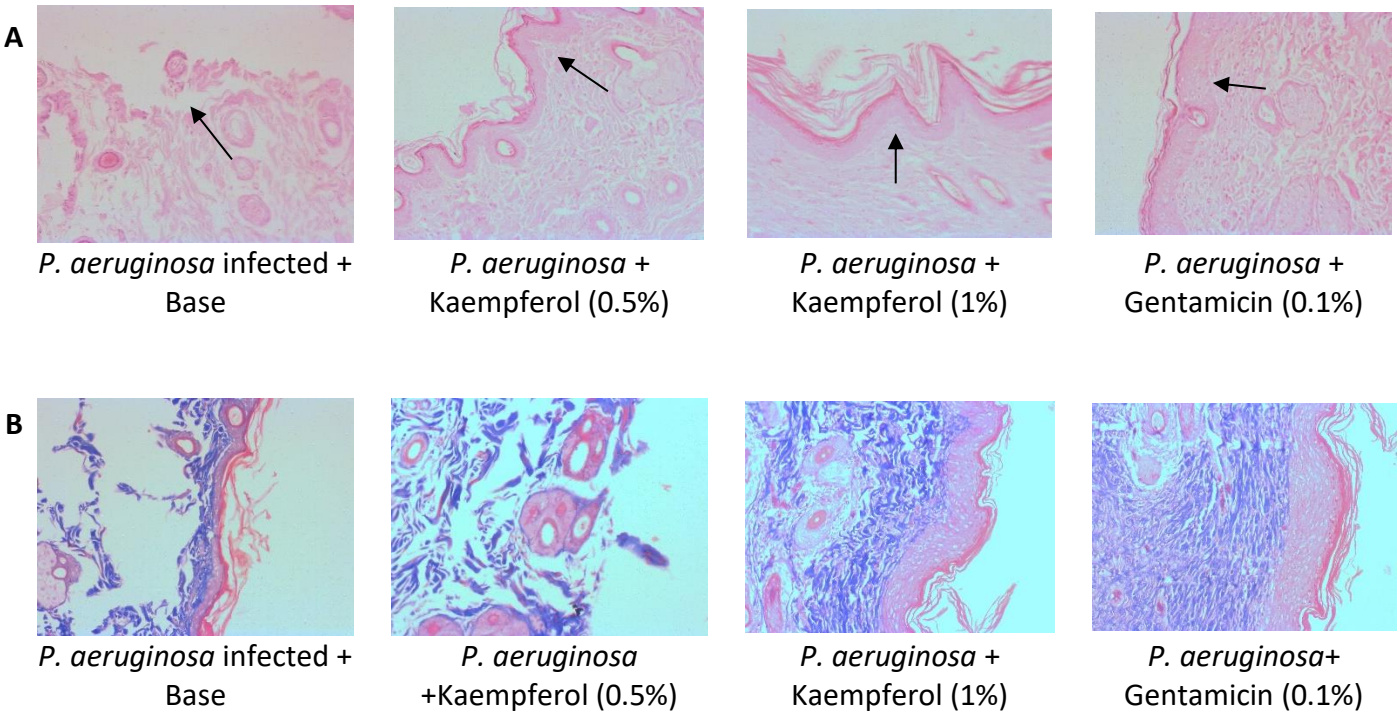

Figure 7. A - Histology of skin after *P. aeruginosa* infection (H&E staining), showing the epidermis (black arrow), capillaries, and inflammatory cells (200×) and B - Histology of skin after *P. aeruginosa* infection (Masson's trichrome stain). The blue coloration indicates collagen deposition (200×).

Similar to the effect observed after MRSA infection, the histological examination of the tissue supported the macroscopic observation. The epithelization was noticeably increased in the antibiotic-treated group followed by the higher kaempferol concentration (1% *w*/*w*) when compared to the control. The epithelial tissue regeneration was less in the control group animals. The lower kaempferol

concentration (0.5% *w*/*w*) had less effect on epithelial regeneration when compared to control (Figure 7a). The effect on collagen deposition was similar to those observed on epithelization. In the base-treated group, the collagen deposition was less compared to kaempferol (1% *w*/*w*) and gentamicin (0.1%), while the low kaempferol dose (0.5% *w*/*w*) showed a moderate collagen deposition (Figure 7b).

Kaempferol increased SOD and catalase activities in a dose-dependent manner (Figure 8). The effect on catalase appeared to be greater than observed on the SOD activity. The standard antibiotic also increased the activities of the enzymes, but its effect was relatively less compared to kaempferol (1%).

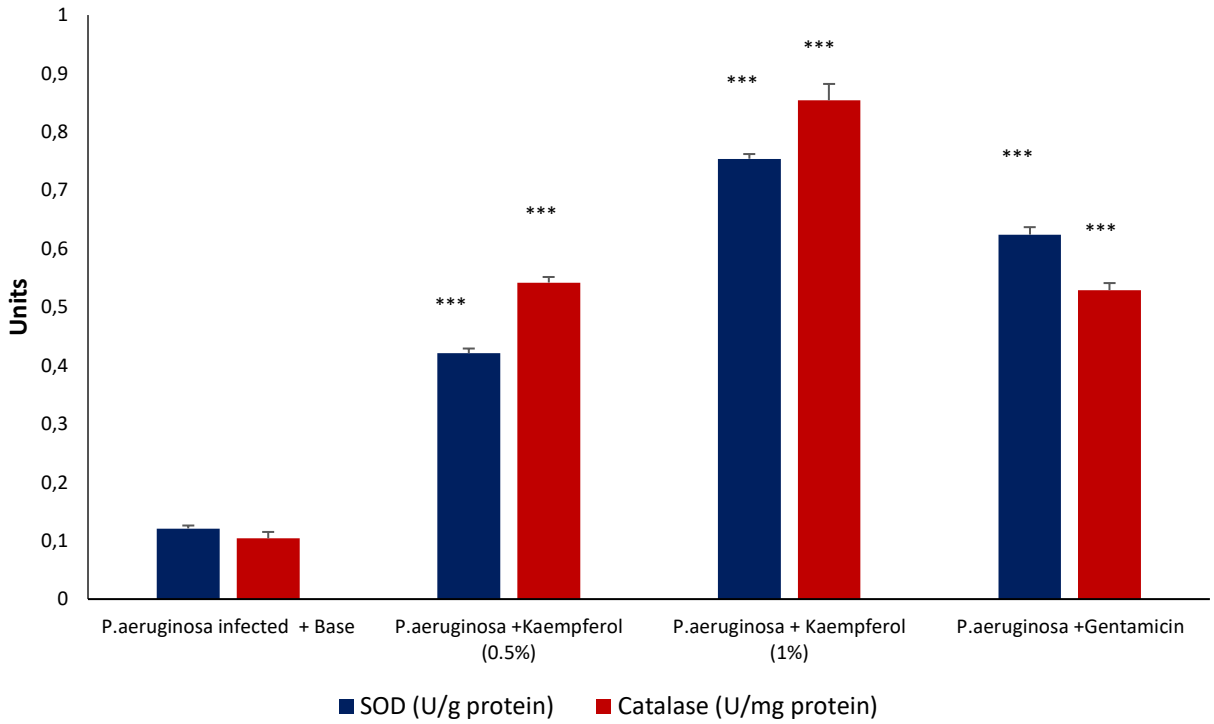

Figure 8. Enzyme activities after *P. aeruginosa* infection. Bars indicate mean ± SEM, n = 6, ***p<0. 01, compared to the base-treated control.

Unlike the effect on bacterial load in MRSA-infected wounds, kaempferol was less effective in reducing the *P. aeruginosa* bacterial load when compared to the control (p <0.05). Gentamicin decreased the bacterial load significantly compared to the control (p <0.01) (Table 5).

Table 5.*P. aeruginosa* count in the wounded tissue.

Treatment	Log_{10} CFU/g of tissue
Control (base)	10.99 ± 1.76
Kaempferol (0.5%*w*/*w*)	8.93 ± 1.98
Kaempferol (1%*w*/*w*)	4.75 ± 1.03*
Gentamicin (0.1%)	3.27 ± 0.78**

Values shown are the mean ± SEM, *p <0.01, **p <0.01 in comparison to the control (base).

Incubation of HaCaT cells with different kaempferol concentrations did not produce any toxic effect on the cells up to 40 µM in the MTT assay. A slight reduction in cell viability was observed at the 80 µM and 100 µM concentrations (Figure 9).

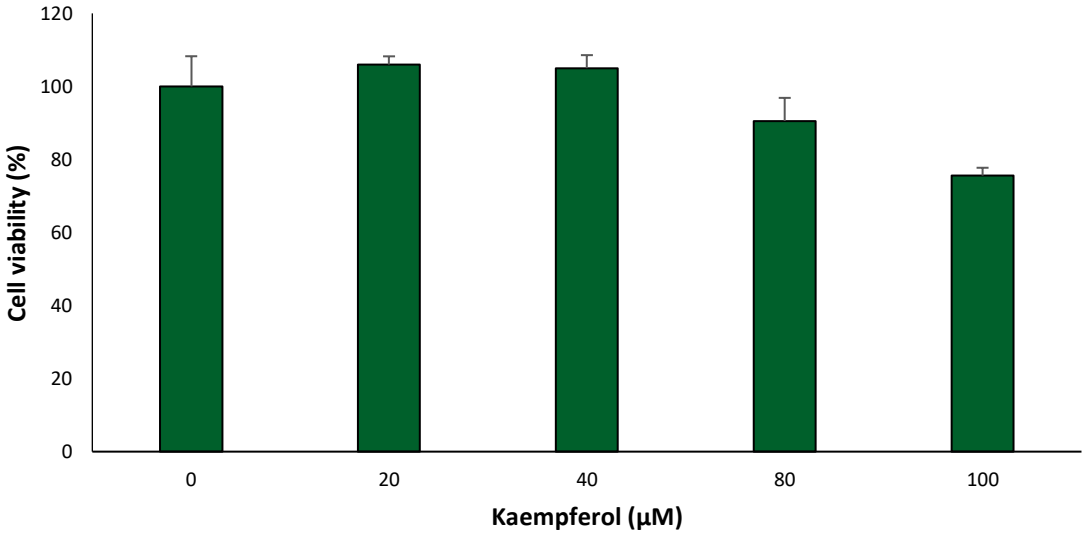

Figure 9. Cytotoxicity assay on HaCaT cells. Bars indicate the mean ± SEM for six trials.

Incubation of HaCaT cells with non-toxic kaempferol concentrations (20 µM and 40 µM) produced no significant change in VEGF or TGF-β1 expression. There was no difference in the expression of these growth factors between the lower and higher kaempferol concentrations, indicating that increasing the concentrations further may similarly lack having a significant effect (Figure 10).

4. Discussion

The effect of kaempferol on controlling bacterial infections and its wound-healing potential were investigated using an excision wound model in diabetic rats. Kaempferol displayed antibacterial effects on MRSA and *P. aeruginosa*. The wound-healing activity was evaluated by macroscopic observations, histological evaluation, antioxidant enzyme activities, and microbial load in the tissues. Regarding the effect on the expression of genes involved in wound healing, VEGF and TGFβ1 were also determined *in vitro* using a HaCaT cell line. In our studies, *M. oleifera* methanolic extract displayed a good wound-healing effect after topical application in both diabetic and non-diabetic infected animals (Al-Ghanayem et al. 2022). Therefore, the same route of administration was followed for kaempferol in the current study. Furthermore, it was reported that extrusion of components using polymer-mediated extrusion formulations of *M. oleifera* preserves phenolic compounds. Therefore, kaempferol may be well preserved in these formulations, showing a better effect than on extrusion using other methods (Park et al. 2022).

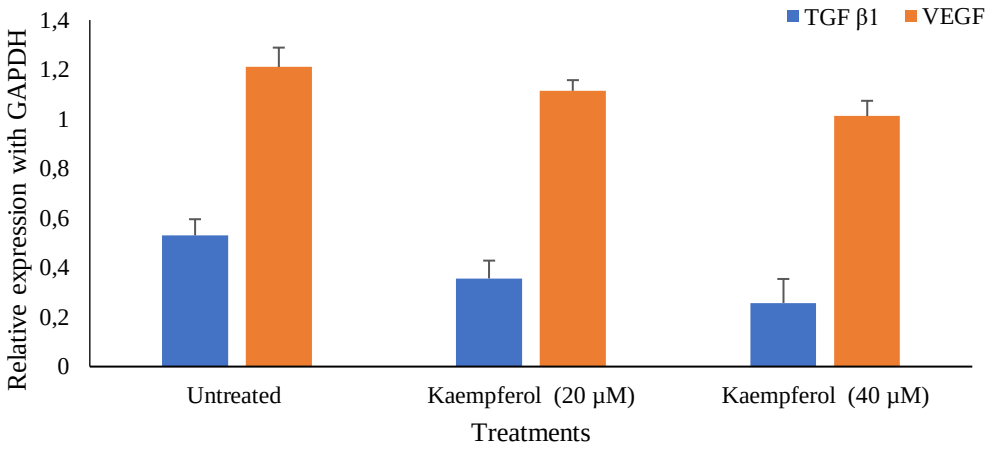

Figure 10. TFG-β1 and VEGF expression in presence of kaempferol. Bars indicate the mean ± SEM for six trials.

Kaempferol is a well-known flavonoid that is reported to display a wide range of biological effects. Some of the extensively studied effects of kaempferol include anti-inflammatory, antioxidant, antidiabetic, and antimicrobial actions (Ren et al. 2019). The wound-healing activity of kaempferol has been reported in both diabetic and non-diabetic rats (Ozay et al. 2019). It is also reported to protect against burn-induced skin injury (Park et al. 2010). However, the mechanism(s) by which kaempferol displays a wound-healing effect is not known.

The management of wound infections is a concern in clinical settings, and a number of antimicrobials are applied to control these infections. Pathogens that infect open wounds and are a challenge to treat were selected based on the literature. Both bacteria chosen are resistant to several conventionally-used antimicrobial agents (Fatima et al. 2022). These pathogens cause nosocomial infections and interrupt wound healing (Serra et al. 2015).

Phytochemicals are being increasingly investigated in the management of wound infections and have been reported to display promising effects (Atef et al. 2019). Furthermore, the management of wound infection in diabetic patients is extremely difficult because diabetes is known to delay the healing process. The diabetic condition reduces blood flow to the tissues by making blood vessels less elastic (Dec-Gilowska et al. 2020). A decrease in blood flow interferes with the wound-healing process. Furthermore, a reduction in leucocyte migration, an essentail mechanism for healing, further delays wound healing (Spampinato et al. 2020).

There are several models available for diabetes induction. We selected a method wherein not all the insulin-secreting cells are destroyed by the cytotoxic agent streptozocin to produce diabetes. In this method, nicotinamide is injected before streptozocin administration. Nicotinamide partially protects the β-cells of the pancreas from the cytotoxic effect of streptozocin, thereby producing type-II diabetes mellitus (Szkudelski 2012). The type-II diabetes model is better than a type-I diabetes model because wound infection, together with severe hyperglycemia in the latter model, may lead to increased mortality among the animals, unless they are injected with insulin to maintain their blood glucose levels. Furthermore, the administration of insulin may influence the normal healing of wounds due to its cell-proliferating effects (Hu et al. 2020). Despite this, in the current study, the infection with *P. aeruginosa* was severe, leading to mortality in the base-treated control group. We did not include a group of uninfected diabetic animals in our study, because the inclusion of such animals would not provide any information about the antibacterial effect of the compounds. This study was performed to confirm whether kaempferol could increase wound healing in infected diabetic animals. Infection in diabetes leads to the development of biofilm and many antibacterial agents were ineffective in the treatment of infected diabetic wounds (Almuhanna et al. 2023). However, the induction of wounds without infection in diabetic animals may not provide any information regarding the activity of kaempferol in treating infection in diabetic wounds. An infected diabetic control is the most suitable group to evaluate the wound-healing effect of compounds in infected diabetic wounds. Furthermore, the determination of the CFU in the wounded tissue reveals the bacterial load at the end of the treatment. This is sufficient to prove the inhibition of bacterial growth or the eradication of the pathogen from the wound.

The results of the current study suggested that kaempferol promotes wound healing through multiple mechanisms. The antibacterial effect of kaempferol is a contributor to its wound-healing action. The evaluation of the *in vitro* antimicrobial effect revealed that it can prevent the growth of the pathogens used in the present study. Similar to several other flavonoids, kaempferol is a known antioxidant when administered orally (Bangar et al. 2022). Wound healing is increased when the free radicals that cause tissue damage are scavenged with the use of antioxidants (Comino-Sanz et al. 2021). The results revealed that kaempferol also has an antioxidant effect after local application, as indicated by the increased levels of the antioxidant enzymes SOD and catalase. This antioxidant effect might have assisted in the wound-healing process in diabetic animals. Earlier studies suggested that it may scavenge free radicals, thereby preventing oxidative damage (Rajendran et al. 2014). It has also been reported that kaempferol increases the expression of antioxidant enzymes in HL-60 cells *in vitro* (Kluska et al. 2022). In the present study, the measurement of SOD and catalase showed that free radicals levels in the wounded tissues were reduced. The mechanism for this effect could be the direct scavenging of reactive oxidative species or the increased expression of endogenous antioxidant in the wounded tissues. In an earlier

study, kaempferol at a concentration of 0.5% was shown to increase the tensile strength of incision wounds through an increased hydroxyproline content after 14 days (Ozay et al. 2019). An increase in hydroxyproline content in excision wounds may also promote the binding of regenerated tissues, thereby supporting the wound-healing process (Nagar et al. 2016). Therefore, this mechanism might also contribute to healing and requires further investigation.

Kaempferol is a known antidiabetic agent that can reduce blood glucose levels when administered orally (Yang et al. 2022). In the current study, kaempferol was applied locally to the wounds, and it can be ruled out that its antidiabetic effect may be responsible for the healing effect. There was no change in the blood glucose levels between the different treatment groups either at the beginning or at the end of the experiment (data not shown).

The histological evaluation of the wounded tissue confirmed the wound-healing action of kaempferol. Increased skin epithelium regeneration and collagen deposition were observed. For the determination of epithelization, sections were stained by H&E staining, while for the observation of collagen deposits, sections were stained using Masson's trichrome stain (Al-Ghanayem et al. 2022). Because the effect on these parameters was similar in both the kaempferol and antibiotic treatment groups, it is difficult to speculate whether the effect was due to antimicrobial/antioxidant effects or through an increase in the expression of pro-healing cytokines. Furthermore, kaempferol is a potent anti-inflammatory agent which has been evaluated in the treatment of both acute and chronic inflammatory diseases (Al-Ghanayem et al. 2022). The induction of wounds and their healing involves inflammation (Schilrreff and Alexiev 2022). The anti-inflammatory effect of kaempferol may be one of the mechanisms involved in wound healing, because earlier reports suggested that kaempferol reduces dermal inflammation in diabetic excision wounds (Ozay et al. 2019). However, the determination of cytokines in the wounded tissue may provide more information regarding its wound-healing action when applied locally.

For the evaluation of *in-vitro* wound-healing effects under diabetic conditions, HaCaT cells were used (Colombo et al. 2017). Kaempferol up to40 µM was non-toxic on HaCaT cells. Diabetic conditions were mimicked *in-vitro* by using Dulbecco's modified Eagle's medium supplemented with high glucose (4.5 g/L). VEGF promotes cell migration, chemotaxis, and vascular permeability (Shibuya 2011), while TGFβ1 is known to stimulate angiogenesis, fibroblast proliferation, and collagen synthesis and deposition (Tracy et al. 2016).Both these cytokines improve the healing of wounds (Al-Ghanayem et al. 2022). Kaempferol was previously reported to have contradictory effects on the VEGF and TGFβ1 actions in the body. A study showed that kaempferol has an angiogenic effect by binding to VEGF (Hu et al. 2020), while another study reported that kaempferol inhibits the angiogenic ability of VEGF (Chin et al. 2018). In the present study, kaempferol did not significantly affect VEGF expression in HaCaT cells. We are unaware of any reports on the effect of kaempferol on TGFβ1 expression, although earlier studies indicated that kaempferol inhibits the effect of TGFβ1 on collagen synthesis (Li et al. 2016). The histological evaluation of the wounded tissues *in vivo* showed an increase in both the number of capillaries and collagen deposition. The *in-vitro* effect on VEGF and TGF-β1 did not support the *in-vivo* effects. This suggested that there may be other mechanisms through which kaempferol increases angiogenesis and collagen deposition. Furthermore, the antioxidant action itself may prevent the effect of reactive oxidative species, and this might indirectly contribute to angiogenesis and collagen deposition (Aneesha et al. 2022). The role of the antibacterial agents on the histological parameters cannot be ruled out, because mupirocin and gentamicin increased both angiogenesis and collagen deposition.

The present study has a few limitations that can be addressed by further studies. Kaempferol was applied locally on the wounds. Investigation of its effect on wound healing due to antidiabetic action should be performed after its oral administration. The wounds were infected using a single pathogen, namely MRSA or *P. aeruginosa*. Wounds are normally infected by more than one organism. The effect of kaempferol on polymicrobial infections may provide a better insight into its efficacy against wound infections observed in clinical settings. Furthermore, this study was limited to an excision wound model, and the effect on other types of wounds, including incision and burn wounds, should also be investigated. The effect on other growth factors, including platelet-derived growth factors, fibroblast growth factors, epidermal growth factors, and insulin-like growth factors, among others, should also be determined. As

indicated above, this study was a continuation of an earlier study on the effect of *M. oleifera* on wound healing. Though kaempferol is an important phytoconstituent of *M. oleifera*, the effect of other phytochemicals present in moringa should also be evaluated to understand the contribution of the constituents to the overall effect of moringa.

5. Conclusions

Kaempferol possesses a good antibacterial effect and increased the wound-healing ability in diabetic rats. The antidiabetic effect and expression of the pro-healing cytokines VEGF and TGFβ1 can be ruled out as contributing factors for its wound-healing effect. The determination of inflammatory cytokines in the wounded tissue in diabetic animals may provide more information.

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