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Wound Healing Activity of Chlorogenic Acid in Diabetic Rats is Mediated Through Antibacterial, Antioxidant, and Proliferative Effects

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Abstract: Chlorogenic acid is known to have antibacterial action against a number of pathogens. This study provides insights into the potentiality of chlorogenic acid in the treatment of chronic infection in wounds. The anti-infective and healing effect of chlorogenic acid in infected wounds was evaluated using diabetic rats. Antibacterial activity of chlorogenic acid against Methicillin-Resistant *Staphylococcus Aureus* (MRSA) and Multidrug-Resistant (MDR) *Pseudomonas aeruginosa* was evaluated *in vitro*. A decrease in the epithelization period ($p<0.001$) and a reduction in bacterial load ($p<0.001$) were observed in the treated groups. In addition to antibacterial activity, chlorogenic acid facilitated wound contraction ($p<0.001$). An increase in antioxidant enzymes was also observed in the wounded tissues ($p<0.001$). A thicker epithelial layer and angiogenesis were observed in the histological evaluation. Chlorogenic acid did not show any cytotoxicity on HaCaT cells *in vitro*. An increase in the expression of proliferative genes Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor-β1 (TGF-β1) was observed in Human keratinocytes (HaCaT) grown in the presence of chlorogenic acid ($p<0.001$). The study confirms that chlorogenic acid supports wound healing with antibacterial, antioxidant, and proliferative effects of pro-healing cytokines genes. This agent may hold the potential for the management of infectious diabetic wounds.

Keywords: Epithelization Period, MRSA, *Pseudomonas aeruginosa*, TGF-β1, VEGF

Introduction

Diabetes mellitus and its complications lead to several health problems including a delay in the healing of the wound, which may end up in the amputation of lower limbs (Rodríguez-Rodríguez *et al*., 2022). The treatment of wound infections becomes difficult due to the hyperglycemic environment and microbial biofilm formation in the wound (Burgess *et al*., 2021). The diabetic wound remains a major clinical task due to its complexity and increased rate of infections with multi-drug resistant pathogens. The wound infection is treated using conventional antibiotics and many of these have a limited effect due to bacterial biofilm and the development of antibiotic resistance (Dos Santos *et al*., 2021). As an alternative to novel synthetic molecules, a large number of bioactive components are considered to have potential antibacterial along with wound healing activities (Khare *et al*., 2021). These bioactive compounds known to promote cellular and tissue regeneration have been reported for wound healing activities even in diabetic conditions (Pawar *et al*., 2021). Agents that possess the capability to attenuate the generation of free radicals, inhibit microbial growth, and increase expression of the proliferative genes involved in wound healing gain are being investigated as potential agents for the treatment of diabetic-infected wounds (Zhang *et al*., 2021).

Chlorogenic acid is a phenolic compound, abundantly available in various plants including tea and green coffee extracts. It is one of the most widely evaluated phenolic acids and is reported to have several biological effects. One of the potent actions of this phytoconstituent is its antioxidant effect which is

reported to protect the heart, kidney, and liver against toxicants (Naveed *et al*., 2018). Chlorogenic acid is known to inhibit several bacteria such as food-borne *Pseudomonas aeruginosa* by increasing the cell membrane permeability in bacteria (Su *et al*., 2019). It is also reported to inhibit the growth of carbapenem-resistant *Klebsiella pneumonia* and prevent quorum sensing suggesting that it can be an effective antibacterial and antivirulent agent (Wang *et al*., 2022). Furthermore, it was reported to have a bactericidal effect against *E. coli* (Kabir *et al*., 2014). In addition to these, chlorogenic acid was also reported to inhibit biofilm formation (Chen *et al*., 2022). All these earlier reports point to its effectiveness against a wide range of bacteria and as an effective antivirulent and antibiofilm agent. Chlorogenic acid is reported to increase the healing of wounds in both normal as well as diabetic rats (Chen *et al*., 2013; Bagdas *et al*., 2015). These earlier reports indicate the good antibacterial effect of chlorogenic acid which may influence wound healing in infectious diabetic wounds.

We investigated *Moringa oleifera* leaf extract for wound healing activity on excision wounds in both diabetic and non-diabetic rats wherein it showed good wound healing activity (Al-Ghanayem *et al*., 2022). Several phytoconstituents were identified in *M. oleifera* in our study and one of the chemical constituents identified was chlorogenic acid. Continuing our investigation further and based on beneficial reports of the wound healing effect of chlorogenic acid in normal rats, the present study was undertaken to evaluate wound healing in diabetic rats. A study on gene expression was also included to explore its cellular proliferative actions.

Materials and Methods

Materials: Chlorogenic acid was purchased from MedChemExpress (# HY-14590, New Jersey, United States). The chemicals used were from standard suppliers. MRSA (ATCC 43300) and MDR- *P. aeruginosa* (ATCC 27853) were used as bacterial pathogens for developing wound infection. Nicotinamide and streptozocin (Hi-media, India). Chemicals for ointment base preparation were from SpecialChem S.A (India).

Preparation of chlorogenic acid ointment: For the ointment base formulation; a 3:6:1 ratio mix of glycol stearate, 1, 2, propylene glycol, and paraffin oil was used (Nayeem *et al*., 2021). To obtain different concentrations, chlorogenic acid 0.5 and 1% (w/w) was added to the ointment base formulation based on earlier reports (Chen *et al*., 2013). The preparation was examined for physico-chemical properties including spreadability and stability using protocols mentioned elsewhere (Nayeem *et al*., 2021). The ointment was subjected to diffusion ability using an agar medium (Jun and Bayoumi, 1986). Thermostability was determined by storing at different temperatures for a minimum of three months (Naira *et al*., 2009).

Antimicrobial activity: The broth dilution technique was used to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). For MBC determination, 10 µL of the broth from clear tubes was streaked onto mannitol salt agar and cetrimide agar media for MRSA and *P. aeruginosa* respectively. The lowest concentration of an antimicrobial agent at which all the bacterial cells were killed was taken as MBC (M100Ed32, 2023).

Animals: Male Wistar rats aged 3.5-4 months old, weighing between 190-210 g were used. Arrival guidelines were followed for handling the animals and for experimental methods (ARRIVE, 2022). The animals were kept for a week in the laboratory for adaptation to a new environment. All the experimental results were compared with control animals that did received only ointment base treatment. The individuals involved in the experiments took maximum precautions to avoid the transmission of bacterial pathogens between animals and all infected animals were isolated. The Ethical Research Committee (ERC) of Shaqra University approved the experimental procedures (No. ERC_SU_20220091).

Skin irritation test: The skin irritation study for the formulation was determined by depilating an area (500 mm^2) on the back of the animal. A higher concentration (1% w/w) of the preparation was applied to the skin and signs of irritation or inflammation were observed every 12 h intervals until 72 h (Nayeem *et al*., 2021).

Streptozocin nicotinamide-induced diabetes: Nicotinamide (120 mg/kg) was administered intraperitoneally after overnight fasting. Streptozocin (60 mg/kg) was injected after 15 min to induce diabetes. Fasting serum glucose was checked after 72 h and animals having serum glucose of more than 150 mg/dL were preferred and selected for further studies (Al-Ghanayem *et al*., 2022).

Wound healing activity: For anesthetizing the rats, an anesthetic cocktail comprising ketamine and xylazine (10:1 ratio) was administered intraperitoneally (1 mL/kg) (Anesthesia, 2022). An excision wound (500 mm^2 area) was made on the dorsal side of the animal after depilating manually using a hair removal cream. After the induction of the excision wound, bacterial pathogens (30 µL) containing 106 CFU/mL were inoculated directly into the wounded area. The animals were grouped into twelve each and five groups were used for each pathogen as follows; group I infected control without treatment; Group II infected with the respective pathogen and treated ointment base (control), group III and Group IV were applied chlorogenic acid ointment at 0.5 and 1% w/w concentrations respectively and group V received either Mupirocin-2% (MRSA) or gentamicin-0.1% (*P. aeruginosa*). Half of the animals from each group were used for the determination of

wound contraction from the 4th day till the 24th day. The wound contraction was measured using a graph sheet superimposed on a transparent sheet. The animals were sacrificed on the 24th day and skin tissues were collected. It was divided into three parts, one part was used to estimate catalase (Link, 1988), Superoxide Dismutase (SOD) (Elstner and Heupel, 1976), the second part for the bacterial load (CFU/g tissue), and the last part for histological analysis. Histological parameters were evaluated by staining tissues with Hematoxylin and Eosin (H and E) stain. The complete wound healing was determined in the rest six animals and this was noted as the epithelization period.

Cytotoxic assay on HaCaT cells and gene expression: MTT assay was employed to detect the cytotoxicity of chlorogenic acid (0-500 µm/mL) on Human Keratinocytes (HaCaT) were grown in Dulbecco's Modified Eagle Medium (DMEM). A study on the expression of VEGF and TGF-β1 genes in the presence of chlorogenic acid was determined as mentioned elsewhere (Al-Ghanayem *et al*., 2022). Chlorogenic acid at safe concentrations (50 and 100 µm/mL) on the HaCaT cell line was used to study its effect on gene expression. The primers used for DNA amplification were used based on our earlier reports (Al-Ghanayem *et al*., 2022).

Statistical analysis: Results are given mean and Standard Error of Mean (SEM). Analysis of Variance (ANOVA) with Tukey's posttest was used to assess statistical significance (SPSS-version 20 for windows).

Results

The chlorogenic acid ointment was stable and homogenous with good diffusion ability, spreadability, and washability. It was free from irritating and inflammatory effects when applied to the intact debilitated skin of rats.

In vitro antibacterial activity: Chlorogenic acid inhibited the growth of both bacteria with a more inhibitory effect on MRSA. The MIC of chlorogenic acid was 128 and 256 µg/mL for MRSA and *P. aeruginosa* respectively. The MBC for MRSA was 512 µg/mL and for *P. aeruginosa*, it was 1024 µg/mL.

Wound healing effect in MRSA-infected excision wound in diabetic rats: The MRSA infection in the wounded tissue manifested as oozing out of fluid exudates and pus formation. Mupirocin and chlorogenic acid at (1% w/w) were effective in healing the infected excision wounds in diabetic rats ($p<0.001$). The healing effect was dose-dependent though no significant difference between the lower and higher concentration was observed. However, the epithelization after antibiotic treatment was the fastest compared to all other treatments (Fig. 1).

There was a significant effect on wound contraction with chlorogenic acid (1% w/w) treatment from day 4 as compared to base-treated control. A significant action on wound contraction after treatment with 0.5% w/w chlorogenic acid was observed from day-8 and this effect was less as compared to chlorogenic acid (1% w/w). Similar to the higher concentration of chlorogenic acid (1% w/w), antibiotic-treated wounds started healing from day 4 (Fig. 2).

The macroscopic observations of the epithelization process were supported by histological examination. The thick epithelial layer of the skin was regenerated after treatment with both concentrations of chlorogenic acid (0.5 and 1% w/w) when compared with the base-treated control (Fig. 3).

The activity of SOD and catalase were evaluated on the 24th day. The antioxidant enzyme activities were significantly more in chlorogenic acid-treated animals in comparison to base-treated control ($p<0.001$). However, the enzyme activities were relatively less after mupirocin treatment when compared with chlorogenic acid (Fig. 4).

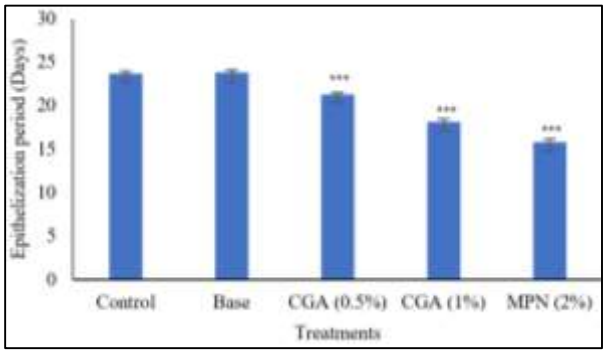

Fig. 1: The epithelization period in diabetic wounds infected with MRSA (positive control-mupirocin 2%). Bars indicate mean ± SEM, n = 6, ***p<0.001 compared to control (base)

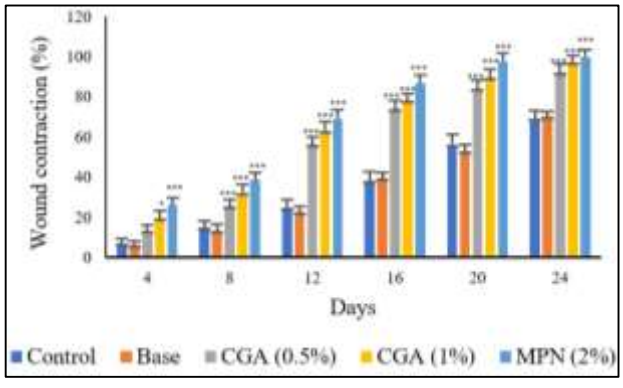

Fig. 2: Wound contraction (%) after MRSA infection. Bars indicate mean ± SEM, n = 6, *p<0.05, ***p<0.001 compared to base-treated control

Fig. 3: Representative images of histological examination and epithelial regeneration in the skin (H and E staining; 200×)

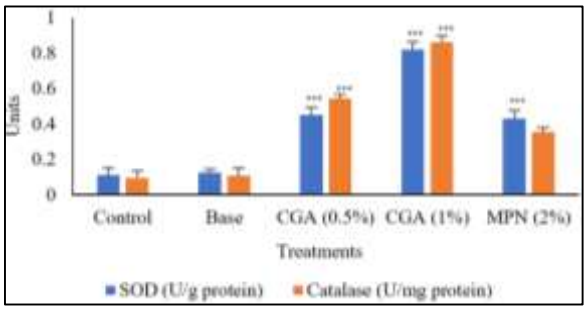

Fig. 4: Effect of chlorogenic acid treatment on the activity of SOD and catalase in the infected wounded tissues. Bars indicate mean ± SEM, n = 6, ***p<0.001 compared to base-treated control

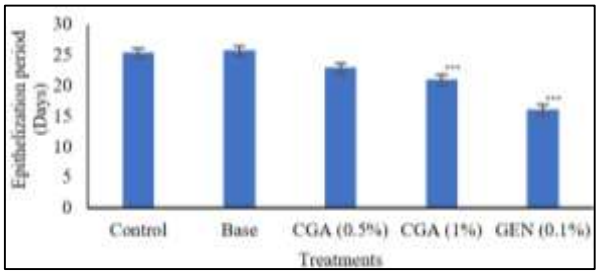

Fig. 5: The epithelization period in diabetic wounds infected with *P. aeruginosa*. Bars indicate mean ± SEM, n = 6, ***p<0.001 compared to base-treated control

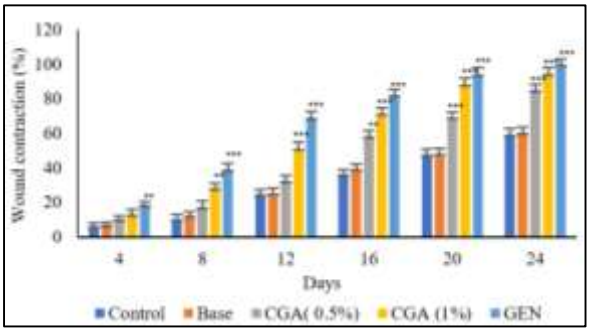

Fig. 6: Wound contraction (%) after *P. aeruginosa* infection. Bars indicate mean ± SEM, n = 6, **p<0.01, ***p<0.001 as compared to the base-treated control

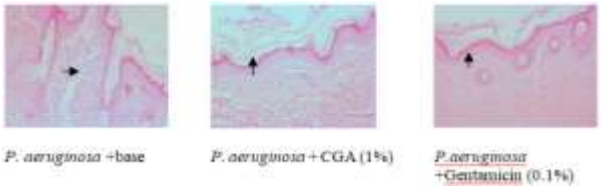

Fig. 7: Representative images of histological examination and epithelial regeneration in the skin (H and E staining; 200×)

Table 1: The MRSA count after different treatments

Group	Log_{10} CFU/g of tissue
Infected control	4.544±0.152
Control (base)	4.477±0.274
Chlorogenic acid (0.5% w/w)	3.397±0.139**
Chlorogenic acid (1% w/w)	2.698±0.145***
Mupirocin (2%)	2.079±0.246***

Values given are mean ± SEM, n = 6, **p<0.01, ***p<0.001 in comparison to the control (base)

The bacterial load on day 24 showed that the even lower concentration of chlorogenic acid (0.5% w/w) decreased the bacterial load significantly (p<0.01) when compared with control. The higher concentration was significantly more effective in inhibiting the bacterial population (p<0.001). The antibiotic, mupirocin (2%) reduced the MRSA load compared to the base-treated control (Table 1).

Wound healing effect after *P. aeruginosa* infection: *P. aeruginosa* infection in wounds was severe and some of the animals were unable to survive. A mortality rate of 33% was observed in both the infected and base-treated control group. The epithelization period was lesser in high-concentration chlorogenic acid (1% w/w) treated animals and antibiotic-treated animals when compared to base-treated controls (Fig. 5). However, no significant effect was observed in the low concentration (0.5% w/w) of chlorogenic acid treated animals when compared to base-treated control. The maximum effect on epithelization was found in antibiotic-treated wounds as expected.

The contraction of the wound was slower in low concentration of chlorogenic acid treated wound (0.5% w/w) when compared to high concentration. The higher concentration of chlorogenic acid (1% w/w) showed a significant effect after the 12th day as compared to the base-treated control. The antibiotic treatment was effective in wound healing from the 4th day onward (Fig. 6).

In histological examination, the maximum epithelial regeneration was observed in antibiotic-treated wounds followed by chlorogenic acid (1% w/w) treated wounds. The epithelial layer was less and thinner in control groups when compared to treated wounds. However, a weaker effect was noticed in low-concentration chlorogenic acid-treated wounds (Fig. 7).

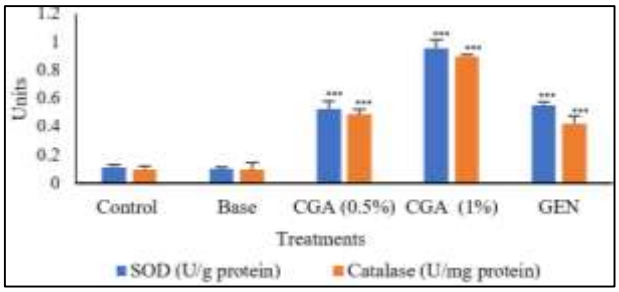

Fig. 8: Enzyme activities after *P. aeruginosa* infection. Bars indicate mean ± SEM, n = 6, ***p<0.001, as compared to the base-treated control

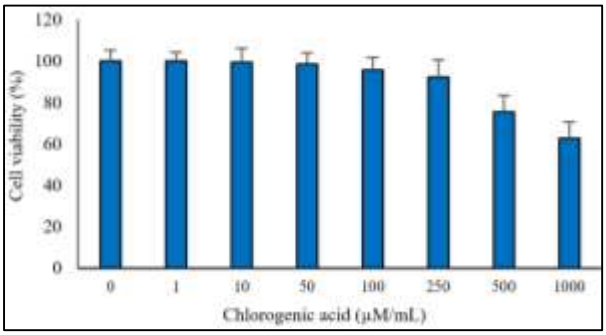

Fig. 9: Cytotoxicity assay of chlorogenic acid at different concentrations on HaCaT cells. Bars indicate mean ± SEM for six trials

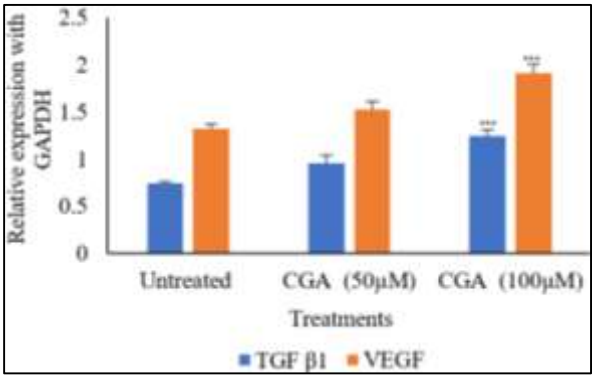

Fig. 10: TFG-β1 and VEGF gene expression in the presence of chlorogenic acid in HaCaT cells. Bars indicate mean ± SEM.***p<0.001 compared to the control

Table 2: The *P. aeruginosa* counts after different treatments

Group	Log10 CFU/g of tissue
Infected control	5.214±0.5478
Control (base)	5.159±0.6890
Chlorogenic acid (0.5%w/w)	2.915±0.3546*
Chlorogenic acid(1%w/w)	1.965±0.4587**
Gentamicin (0.1%)	1.245±0.3784***

Values shown are mean ± SEM, *p<0.01, **p<0.01,***p<0.001 compared to the control (base)

The treatment of wounds with chlorogenic acid and the antibiotic increased the SOD and catalase in the tissues as compared to the control with a maximum effect on antioxidant enzymes in chlorogenic acid (1%) treated wounds. The effect was dose-dependent but the difference between the lower concentration of chlorogenic acid (0.5% w/w) and higher concentration (1%) of the chlorogenic acid was non-significant (Fig. 8).

The bacterial load in *P. aeruginosa*-infected wounds was significantly reduced with gentamicin (0.1%)treatment followed by a high concentration of chlorogenic acid (1% w/w) in comparison with the base-treated control. In contrast with MRSA count, chlorogenic acid had a lesser effect in reducing *P. aeruginosa* count (Table 2).

The cytotoxic evaluation of chlorogenic acid by MTT assay revealed that chlorogenic acid is non-toxic on HaCaT cells. Cell viability of above 90% was observed at 250 µm/mL concentration. A cell viability of above 60% was observed at 1000 µm concentration (Fig. 9).

In HaCaT cells, the expression of VEGF and TGF-β1 genes was significantly increased in the presence of chlorogenic acid (100 µm/mL). The effect was dose-dependent but the lower concentration of chlorogenic acid (50 µm/mL) was ineffective in altering the gene expression. There was no significant difference between the chlorogenic acid (0.5% w/w) and chlorogenic acid (1%) (Fig. 10).

Discussion

The outcome of this study indicates that chlorogenic acid promotes the healing of infected wounds through anti-infective, antioxidant, and proliferative effects in diabetic rats. Chlorogenic acid is abundantly present in several plants including vegetables, beverages, and fruits. Earlier studies on the antioxidant action of chlorogenic acid suggest that it is a potent antioxidant agent (Naveed *et al*., 2018). As mentioned above, there are several reports on the antimicrobial effect of chlorogenic acid (Su *et al*., 2019; Wang *et al*., 2022; Kabir *et al*., 2014; Chen *et al*., 2022). Furthermore, chlorogenic acid is also reported to increase the healing of uninfected wounds in both normal and diabetic rats (Chen *et al*., 2013; Bagdas *et al*., 2015). This study is the first attempt to determine the effect of chlorogenic acid in infected diabetic wounds. The bacteria selected in this study are reported for opportunistic infections clinically and impair the healing of wounds, in normal as well as diabetic patients (Macdonald *et al*., 2021). Hence, this study revealed the potential of chlorogenic acid in wound healing and infection control in infected diabetic wounds.

Chlorogenic acid is a polyphenol consumed by humans in their diet. It is known to influence cell activity through the modulation of inflammation and metabolic processes. This has generated interest in its anti-

inflammatory and wound-healing activity (Lee *et al*., 2021). Chlorogenic acid was shown to reverse the UVA irradiated suppression of TGF-β1 expression (Xue *et al*., 2022). The results also supported the antioxidant effect of chlorogenic acid in diabetic-infected wounds. Similar to earlier reports on normal skin, an increase in TGF-β1 expression was observed in the infected skin of diabetic animals. The base was selected based on the physicochemical properties of the prepared ointment. Several different bases mentioned in the pharmacopeias and earlier references were screened and the base with maximum stability, diffusion ability, spreadability, etc., was selected.

Infection of wounds in diabetic animals led to a grayish layer over the wounded area with pus formation indicating the development of biofilm over the wounds. Healing of these biofilm-formed wounds by chlorogenic acid confirms its antibiofilm effect that has been reported earlier (Chen *et al*., 2022; Wang *et al*., 2022). Earlier reports on the antimicrobial activities were reported against different species of non-resistant bacteria. The strains of bacteria used in the present study were highly resistant to many conventionally used antibiotics. The inhibitory effects of chlorogenic acid against these pathogens indicate its effectiveness against multidrug-resistant pathogens. However, it has to be noted that the MIC of chlorogenic acid reported earlier for non-resistant pathogenic bacteria was at a much lower concentration than that obtained in the present study (Lou *et al*., 2011). The antibacterial effect though moderate reduced the bacterial count in the wounded tissues significantly. As expected, more effect was observed on MRSA as compared to *P. aeruginosa* due to the composition of the cell wall and resistant pattern (Koohsari *et al*., 2015).

Apart from the antimicrobial effect, the faster healing of wounds is facilitated due to the antioxidant action. Chlorogenic acid is a potent antioxidant agent and it is reported to scavenge free radicals involved in skin damage by increasing the activity of SOD, catalase, glutathione, and reduction of lipid peroxidation (Chiang *et al*., 2015). Hyperglycemia is known to delay wound healing through various pathways that include the generation of free radicals. The increased healing observed may at least be in part due to the antioxidant effect (Burgess *et al*., 2021)

Chlorogenic acid was safe on HaCaT cell lines. This result supports the earlier study that it protects HaCaT cells from radiation-induced oxidative damage (Cha *et al*., 2014). It is also reported to increase the expression of TGF-β1 and enhance collagen deposition in normal skin (Chiang *et al*., 2015). A thicker epithelium and an increase in capillaries were observed after histological evaluations. The *in vitro* evaluations in gene expression especially, VEGF and TGF-β1also supported *in-vivo* results of wound healing. The results suggest that a multifaceted action of chlorogenic acid is involved in wound healing and controlling the infection in diabetic wounds.

Conclusion

Chlorogenic acid showed an antibacterial effect against MRSA and *P. aeruginosa* in both *in-vivo* and *in vitro* experiments. It was more effective in controlling MRSA infection and moderately in controlling *P. aeruginosa* in the infected diabetic wounds. The present study revealed that chlorogenic acid supports wound healing in infected wounds through anti-microbial, antioxidant, and proliferative action on genes. Evaluating the inflammatory cytokines in the wounded tissue may provide more evidence about the involvement of anti-inflammatory action in the wound-healing effect of chlorogenic acid.

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Author's Contributions

Abdullah Abdulrahman Al-Ghanayem, Mohammed Sanad Alhussaini and AbdulRahman Abdulla Ibrahim Alyahya: Participated in all experiments, coordinated the data analysis, and contributed to the written of the manuscript.

Mohammed Asad and Babu Joseph: Designed the research planned, and organized the study.

Ethics

The research protocol was reviewed and approved by the ethical research committee of Shaqra University (approval number ERC_SU_20220091). The methods used in the current study were standard methods and were in accordance with the arrive guidelines.

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