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Wound Healing Potential of *Calligonum Comosum* in the Rat Model: Morphological and Histopathological Evidences

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Abstract *Calligonum comosum* Family Polygonaceae is a perennial shrub that grows in the desert areas in Saudi Arabia. The plant is extensively used for economic purposes and in Saudi traditional medicine. The plant is used for tanning hides and for wound. The current study aims to provide scientific evidence for the wound healing potential. The plant was extracted with methanol at room temperature and with water by decoction against two Gram-positive bacterial strains; *Bacillus subtilis* and *Staphylococcus aureus*, two Gram-negative bacterial strains, *Escherichia coli* and *Klebsiella pneumonia*, in addition to a pathogenic fungus *Candida albicans*. The methanol and water extract were formulated as creams and applied to evaluate the wound healing potential using rats as experimental animals. The methanol extract was more active in the antimicrobial testing and in accelerating the healing of wounds than the water extract. Wound healing potential was accessed via morphological parameters such as wound contraction, epithelization period and detailed as well as histopathological study of the healed skin. The wound was completely healed in the control group treated with Fucidin 2% cream after 18 days, while the methanol extract treated group reached 96.6% wound contraction in the same period. The water extract was much less active and in 18 gays resulted in only 73.2% wound contraction. Skin samples of methanol extract treated rats showed much improvement and almost normal appearance with normal content of collagen fibers, although slight weakness near the epidermis layer was observed. The obtained results strongly document the beneficial effects of *C. comosum* as wound healing activator and reveal the best method for the extraction of the plant.

Keywords *Calligonum comosum*, Wound Healing, Histopathology, Rats

1. Introduction

Calligonum L. genus includes 80 species of xeromorphic shrubby herbs that are widely distributed throughout Southern Europe, Western Asia, and Northern Africa [1]. *Calligonum comosum* is an evergreen woody plant distributed in the sandy soiled Arabian Sahara [2]. The traditional use of *C. comosum* in folk medicine to treat diseases such as hepatotoxicities, polycystic ovary, and osteoporosis is evident [3]. Further, the plant has been utilized by local homeopathic healers for stomach illness, toothache [4] and as a stimulant and an astringent. *C. comosum* is reported to possess cytoprotective [4] and hypoglycemic [5] effects. Besides, anthraquinones of *Calligonum* species showed high antimicrobial potential [6]. Bedouins use the leaves in tanning of the animal's skin and it is also known to have activity against some stomach problems and skin wounds [7].

Assays have been conducted to study the efficacy of the total extract and various fractions of *C. comosum*. For instance, ethyl acetate fraction showed strong potency as an anticancer agent using Ehrlich ascites (Ehrlich cells), brine shrimp and antioxidant assays [8]. *C. comosum* extract exerted anti-inflammatory, anti-proliferative and anti-angiogenic effects on endometriotic lesions [9]. *C. comosum* organic extracts were found effective to control the growth of *Listerial* species causing food contamination [10]. Several phytochemicals are produced by this herb including steroids, phenolics, tannin, flavonoids, alkaloids, terpenoids and saponins [11].

As the plant is used in *the* tanning of hides and possesses antimicrobial activity [12], the current work explored the possible wound-healing enhancing effect of the methanol and water extracts of *C. comosum*.

Figure 1. Photographic representation of wound area on 0th day.

2. Materials and Methods

2.1. Plant Material

Calligonum comosum L'Hér. aerial parts were collected from An Nafud Desert, Al-Tharwairat valley (25.7109523, 44.3859773) west of Al Qassim region in Dr Saniya Kamal, Prof. of Taxonomy, Department of Botany, Faculty of Science, Alexandria University. A voucher specimen # 19420 was *deposited* at the herbarium of the Faculty of Science, Alexandria University.

2.2. Extraction and Fractionation

The shade dried plant materials (15 days in controlled temperature of 22℃ and continuous aeriation) were powdered and 1 kg was extracted with methanol by maceration at room temperature (3 X 4L). The combined extract was evaporated to dryness using Rotary Evaporator under Vacuum and reduced temperature to give (103 g) methanol extract (ME). Another 200 g of the plant material was extracted with distilled water by boiling under reflux for 4 hrs. The resulting extract was lyophilized to dryness using Freeze dryer to produce 24 g water extract (WE).

2.3. Antimicrobial Activity

The antimicrobial activities of different extracts and fractions were explored against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumonia* as well as the fungus *Candida albicans*. The Microbiology Research Lab, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj was the source of the used strains. The bacteria were sub-cultured onto Mueller-Hinton agar for 24 h, while the fungal strain *C. albicans* was sub-cultured for 5 days at 37℃ using Sabouraud-dextrose agar. The sub-cultured plate's colonies were then grown in M-H broth and S-D broth for antibacterial and antifungal assays, respectively, to comply with the 0.5 McFarland turbidity standard equivalent to 1.5-108 CFU/mL. The agar-well diffusion method was applied [13]. The petri dishes were prepared by using molten-cooled agar (15 mL). From each test microorganism 0.1 mL of inoculum was spread onto the agar and the agar was allowed to solidify under sterile conditions. Sterile cork-borer was used to punch 6-mm diameter well. On testing, the wells were filled with 100 µl of the tested samples in DMSO in triplicates. The plates were refrigerated for 30 min prior to incubation for proper diffusion of the tested samples into agar layer. The plated were incubated for 24 h at 37℃ along with the negative control of DMSO. The produced clear inhibition zones were measured in mm.

2.4. *In vivo* Experiments

2.4.1. Experimental animals

Male Wistar rats weighing between 180–200 were bred and housed in the lab animal unit, College of Pharmacy; University of Prince Sattam bin Abdulaziz in ventilated cages (Rat IVC Blue Line, Techniplast, Buguggiate VA, Italy). Animals were maintained in controlled environmental conditions of 25±1℃ and 12 h/12 h light/dark cycle with feed and water *ad libitum*. The guidelines for use of animals [14] were followed in care and handling of rats. The experimental procedures were approved by the Bioethical Research Committee (BERC), Prince Sattam bin Abdulaziz University (ref No. BERC-008-04-21).

2.4.2. Preparation of creams for wound healing activity

The topical creams were made by melting petrolatum (21.16 g) min a water bath at 70 °. The aqueous phase, containing water (17.17 mL) and sorbitan monolaurate (5 g) as a surfactant, was mixed with glycerol (4.67 g) and 2.5 g of either the ME or WE. The oily phase that contains tween 80 surfactant (2 g), The oily phase containing tween 80 surfactant (2 g), was gradually added to the aqueous phase with continuous stirring using kitchen mixer (Kenwood, USA) at 500 rpm. The cream was removed from water bath and cooled down after the mixture was stirred for another 5 min [15].

2.4.3. Experimental design

ME and WE creams at 5% were tested for their wound healing potential using excision wound [16]. Twenty rats were anesthetized using ketamine hydrochloride (50 mg/kg, ip). The skin of the dorsal area of each rat was shaved using an electrical clipper followed by disinfection with 70% alcohol. A uniform wound of 11 mm in diameter was excised from the shaved dorsal region of each rat with the aid of sterile toothed forceps and sharp pointed scissors. Animals were randomly divided into four groups, with 5 rats per group. The first group was treated topically with the cream base as a negative control group (NC); the second group was treated topically with 2% Fucidin cream as a reference group (REF); the third group was treated topically with 5% ME cream; and the fourth group was treated topically with 5% WE cream.

Treatment samples were applied topically over the entire wound area once daily starting from wounding day (day 0) until complete healing was observed. The wound area was measured every 6 days by tracing its margins with the help of transparent sheet. These wound tracings were retraced on a piece of mm-scale graph paper. Wound contraction as a percentage reduction in the wound area with respect to the initial wound area was calculated from the formula below [17].

$$\%\ \text{Wound Contraction} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \times 100$$

where n = number of days [6th, 12th, 18th and 24th day].

Epithelization period was monitored by observing the number of post wounding days (pwd) required for Escher to fall away, leaving no raw wound behind [18].

2.4.4. Histopathological evaluation of wound tissues

After animal scarification, sample tissues from wound sites were removed and fixed in 10% buffered formalin, blocked with paraffin and then sectioned into 5 μm thickness using rotary microtome (SHUR/Cut 4500, TBS, NC, USA) [19]. Hematoxylin and eosin (H&E) dye was used as the general staining method [20] and Masson trichrome was used to analyze connective tissue fibers [21]. Sections were analyzed using a light microscope (Olympus BX-50 Olympus Corporation, Tokyo, Japan).

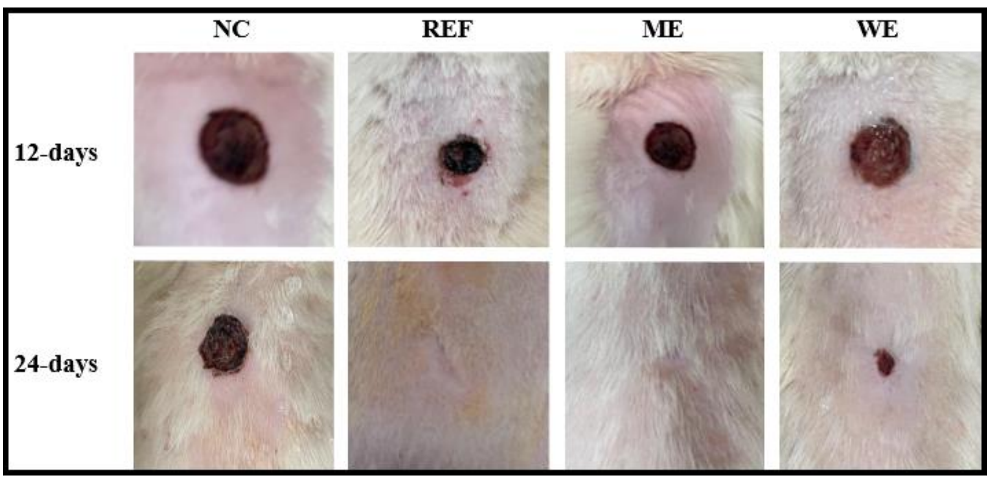

Figure 2. Photographic representation of wound area following topical application of 2% Fucidin cream (REF) and 5% ME and WE creams in rats on the days 12 and 24 of treatment.

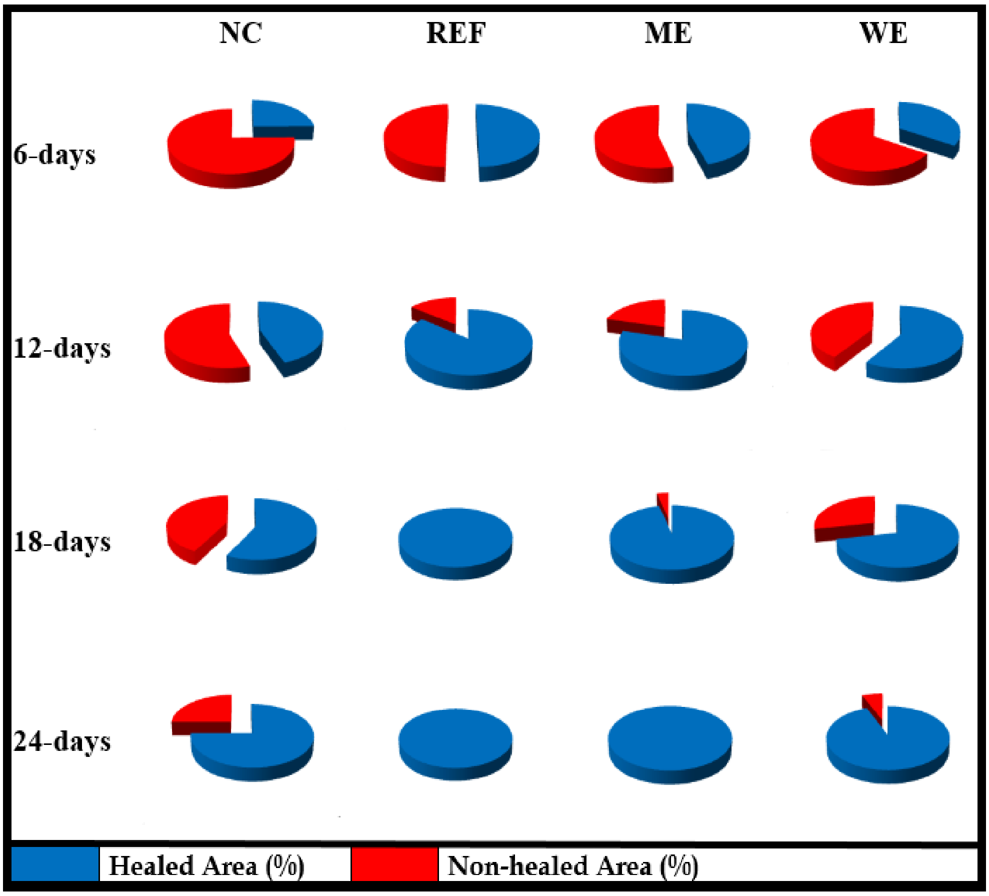

Figure 3. Effect of ME and WE creams on the percentages of wound contractions in rats

Table 1. Inhibition zone (mm) of extracts and fractions of *C. comosum* at 50 mg/mL

	Staph. aureus	*B. subtilis*	*E. coli*	*k. pneumonia*	*C. albicans*
ME[a]	24	21	12	25	NA[b]
WE[a]	15	14	NA	14	NA

[a] tested at 100 mg/mL.

[b] Non active.

Table 2. Effect of ME and WE creams on the percentages of wound contractions and epithelialization periods in rats

Groups	Mean (%) of wound contraction ± SEM				Period of Epithelization (pwd)
	6th day	12th day	18th day	24th day	
NC	28.6±1.63	47.4±2.04	59.8±1.66	76.2±1.59	29.4±1.36
REF	52.4±2.23●	86.6±1.96●	100.0±0.00●	100.0±0.00●	19.2±0.97●
ME	48.6±3.63●	80.4±1.96●	96.6±1.69●	100.0±0.00●	20.6±0.68●
WE	37.4±2.50●#	61.2±2.27●#	73.2±1.46●#	94.2±0.37●#	25.6±0.93●#

Values are expressed as mean ± S.E.M., n =5 rats/group.

● indicate significance compared to negative control group at $p < 0.05$.

indicate significance compared to reference group at $p < 0.05$.

3. Results and Discussion

Antibacterial and antifungal activities of extracts and fractions against pathogenic bacteria and fungi were investigated by the Agar well diffusion method. These activities were assessed by comparing sizes of inhibitory zones, including diameters of wells, on the agar surface around the wells. Values of <8 mm are considered inactive against microorganisms (Table 1). Antimicrobial Activity testing results using Gram-positive, Gram-negative bacteria and one fungi (Table 1) showed the absence of any activity against the fungus *C. albicans*. Generally, both *staph. aureus* and *k. pneumonia* were the most sensitive organisms.

On the day of wound induction, a circular wound with 11 mm diameter was created (Figure 1). At that time, no significant differences in wound sizes were observed between all groups of rats. The reductions of the wound size in the different groups over a period of 24 days are shown in Figures 2 & 3 and Table 2. Rate of wound contraction in NC group was 24.84% to 44.63% from day 6 to day 12 and 57.68% to 74.95% from day 18 to day 24. The average time in days required for the shedding of eschar without leaving any residual raw wound in this group was 29.4 days.

In excision wound, the REF and ME creams showed faster wound contraction compared with the NC group. Further, the mean epithelization periods observed with REF and ME-treated groups were 19.2 and 20.6 days, respectively. WE-treated group showed less wound healing activity compared with the REF group.

Wound hallmarks include the absence of epithelial integrity and abnormal structures and functions of skin and its underlying tissues [22]. Wound healing is a complex restoring process of damaged tissue aiming to restore the cellular structures and tissue layers integrity closer to the original state [23]. Wound contraction occurs during this process to accelerate wound closure and restore functional barrier which in turn facilitate re-epithelialization of the wounded skin [24].

Epithelization period, which is the time required for proliferation and migration of epithelial cells across the wounded bed, of the excision wound is an important parameter to assess the wound healing process [25]. In the present study, the epithelization period observed with topical application of ME was 20.6 ± 0.68 days while the period observed with WE was 25.6 ±0.93 days (Figure 4). ME treated rats, thus, showed a faster healing rate which was comparable with REF treated group (19.2 ±0.97days). In this regard, it has been shown that shorter epithelialization periods with enhanced wound contraction reflect the great wound healing activity of any tested drugs [26].

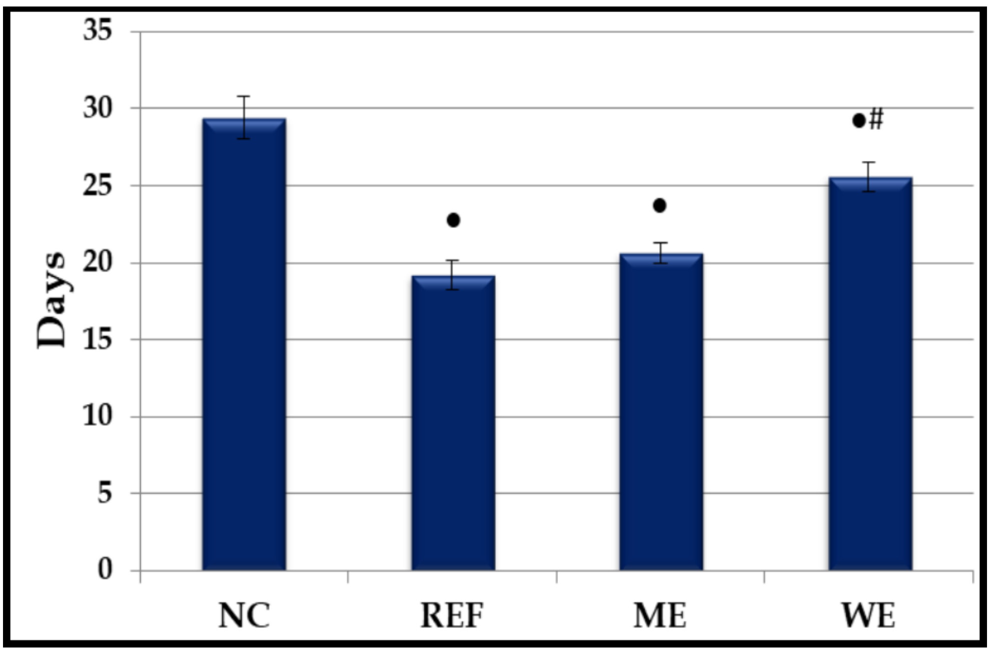

Figure 4. Effect of ME and WE on the epithelialization periods in rats.

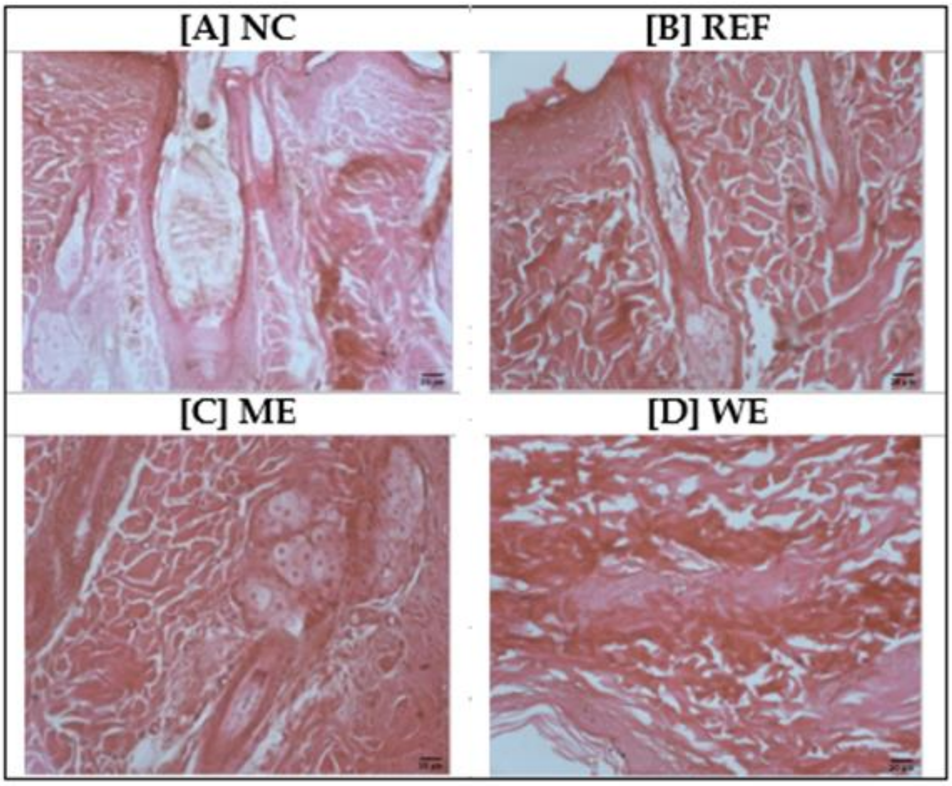

Figure 5. Histological sections stained with H&E of the wound tissue of rats after topical application of cream base (A), Fucidin cream (B), ME cream (C) and WE cream (D). Magnification: x400.

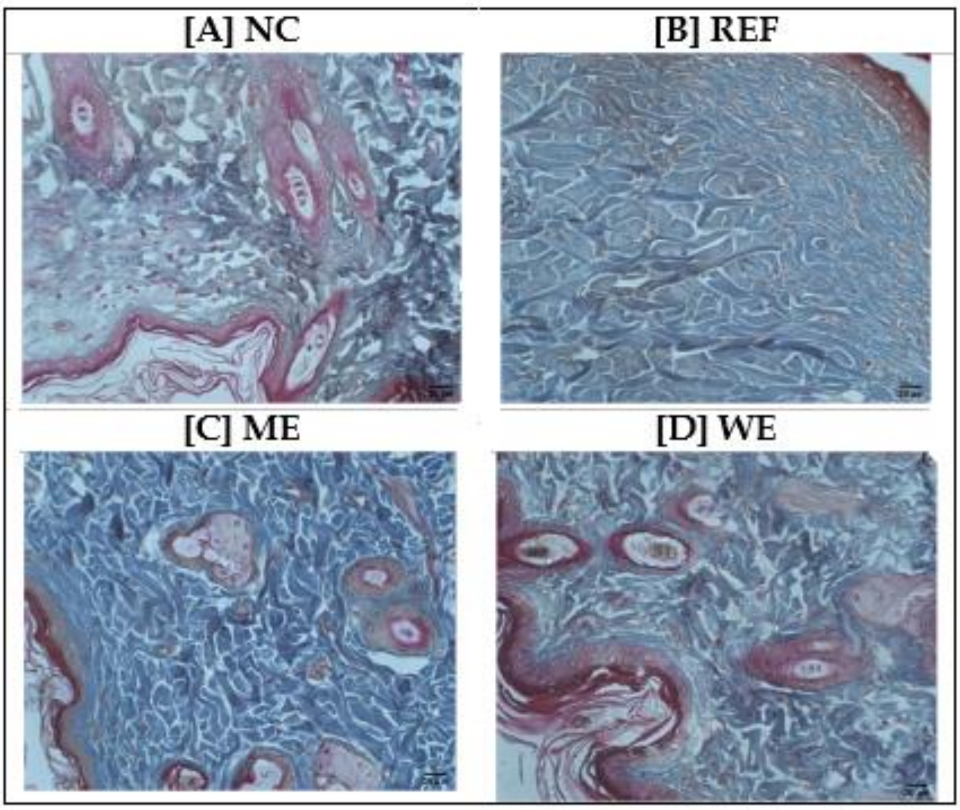

Figure 6. Histological sections stained with Masson-Trichrom of the wound tissue of rats after topical application of cream base (A), Fucidin cream (B), ME cream (C) and WE cream (D). Magnification: x400. Step 3: Copy all styles entitled with HRPUB to the Normal.dotm and close the Organizer (Figure 4)

Micrographs of Hematoxylin and Eosin (H&E) stained tissues are indicated in Figure 5 and those stained with Masson-Trichrom are presented in Figure 6. H&E staining was used to evaluate the inflammatory response; dermal closure and remodeling of the wound area, while Masson-Trichrom stain was used for selective collagen and collagen fibers staining. Histological examination of skin samples of NC group showed multiple areas of tissue damage, degeneration and wide areas of necrosis (Figure 5-A). Further, skin of NC rats showed multiple areas of losing collagen fibers (Figure 6-A). Histopathological evaluation of REF group showed healing of the defect area (Figure 5-B) with normal content and distribution of collagen fibers (Fig. 6-B). Micrographs of ME-treated rats revealed much improvement and showed a close similarity to the REF group (Figure 5-C). The healed ME-treated tissues showed normal content of collagen fibers with slight weakness near the epidermis layer (Figure 6-C). Skin of WE-treated rats showed slight improvement and still suffered from tissue damage, degeneration, and necrosis (Figure 5-D) in addition to areas of losing collagen fibers (Figure 6-D).

Collagen plays an important role in wound healing process as controlled synthesis, deposition, and maturation of new collagens largely determine the ultimate consequences of this process [27]. In addition, collagen is considered as a key extracellular protein that affects wound strength and integrity of tissue matrix [28].

C. comosum composed of a variety of phytochemicals including flavonoids, alkaloids, tannins, terpenoids, phenolics and essential oil [8, 10]. In our study, we believe that the presented wound healing potency of ME might be attributed to its phenolics and flavonoids contents. In fact, these phytochemicals show strong wound healing properties through multiple mechanisms such as wound contraction, decrease epithelialization period, and prevent secondary bacterial infection, as well as its astringent and anti-inflammatory effects [4, 8, 10]. Our histopathological findings that wound contraction of ME-treated group has better venerability of collagen synthesis. This could also be attributed to the presence of phenolic compounds [29].

4. Conclusion

Two methods of extractions were applied to the plant materials. The ME was more active than the WE in the tested antimicrobial activity. ME and WE were evaluated for their potential to accelerate wound healing. The epithelialization period of the excision wound in ME-treated rats (19.6 ± 1.72 days) was comparable to that of the reference Fucidine cream-treated group (15.7 ± 1.15 days). The study suggests that 5% ME cream has a potential benefit in enhancing the wound healing process. The mechanism of action of ME as wound healing accelerator was postulated to involve epithelization and wound contraction. Skin samples of ME-treated rats showed much improvement and almost normal appearance with normal content of collagen fibers, although slight weakness near the epidermis layer. This effect may be attributed to the phenolic contents of the plant.

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