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ORIGINAL ARTICLE

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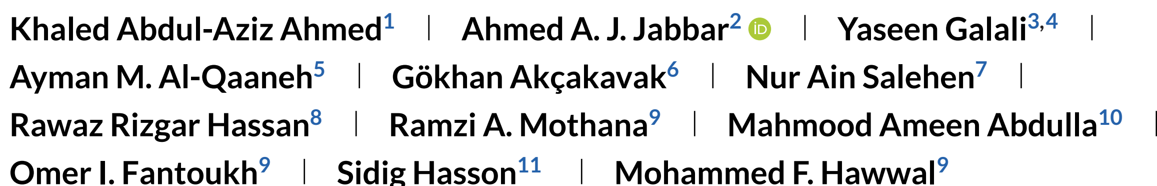
Cumin (*Cuminum cyminum* L.) seeds accelerates wound healing in rats: Possible molecular mechanisms



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Abstract

Wound healing is a complex, intricate, and dynamic process that requires effective therapeutic management. The current study evaluates the wound healing potentials of methanolic extract of *Cuminum cyminum* L. seeds (CCS) in rats. Sprague Dawley (24) rats were distributed into four cages, wounds produced on the back of the neck, and received two daily topical treatments for 14 days: A, rats received normal saline; B, wounded rats treated with intrasite gel; C and D, rats received 0.2 mL of 250 and 500 mg/kg of CCS, respectively. After that, wound area and closure percentage were evaluated, and wound tissues were dissected for histopathological, immunohistochemical, and biochemical examinations. Acute toxicity trials of methanolic extract of CCS showed the absence of any physiological changes or mortality in rats. CCS application caused a significant reduction in wound size and a statistically elevated percentage of wound contraction than those of vehicle rats. CCS treatment caused significant up-regulation of collagen fiber, fibroblasts, and fewer inflammatory cells (inflammation) in granulation tissues. TGF-β1 (angiogenetic factor) was significantly

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more expressed in CCS-treated rats in comparison to normal saline-treated rats; therefore, more fibroblasts transformed into myofibroblasts (angiogenesis). CCS-treated rats showed remarkable antioxidant potentials (higher SOD and CAT enzymes) and decreased MDA (lipid peroxidation) levels in their wound tissue homogenates. Hydroxyproline amino acid (collagen) was significantly up-regulated by CCS treatment, which is commonly related to faster wound closure area. The outcomes suggest CCS as a viable new source of pharmaceuticals for wound treatment.

KEYWORDS
antioxidants, *Cuminum cyminum* L., histology, immunohistochemistry, wound

1 | INTRODUCTION

Skin is well known as the largest human organ that constitutes about 3/20 fraction of adult body weight. The skin is considered the layer between two separate environments (body and outside environment), maintains body temperature, and prevents water loss, and microbial penetration.[1] Skin as in the case of most organs composed of outer epidermis contains keratinocytes, Langerhans cells, and melanocyte cells; the dermis (middle layer) contains immune cells, blood vessels, fibroblasts, extracellular matrix (ECM), and skin appendages that nourish and supports the skin; hypodermis (innermost layer) contains numerous adipocytes, stores energy, aids in dermal growth, serves as connector between the skin layers and the connective tissue below it.[2] Skin is composed of dense lymphatic vessels and lymph capillaries that are innervated horizontally below the epidermis, followed by collector vessels deep down in the dermal layer and collecting lymphatic vessels in the subcutaneous fat layer. The lymphatic vessels are joined with local lymph node drainage of the skin and then connect with the regional sentry lymph nodes before reaching the thoracic duct.[3] Although human skin can repair itself spontaneously to regain its structure and functional characteristics, wound care and management remain an important necessity to avoid infection, ameliorate pain, and desiccation, protect the wound area, accelerate skin tissue repair, and avoid scars, mainly in chronic, wide open, and burn wounds.[4] The data record of 2014 revealed 17.2 million acute wound cases visiting US hospitals.[5] Recent statistics showed that about 1%–2% of the people living in industrialized countries (more developed) have chronic wounds.[6] The duration and cruelty of the wound healing process be can associated with numerous factors, patient's history of diseases, aging, diabetic ulcers, pressure ulcers, inappropriate treatment, and vascular ulcers, which were also correlated with increased morbidity and mortality rates. The global industrial market for wound products is rapidly rising reaching almost 12$ billion in 2020 and is expected to reach 18.7$ billion by 2027.

The pharmaceutical production provides numerous drug synthetics for wound management and wound contraction, but most of them does not fulfill desired intention and often come with many side effects. While, natural products and plant-derived active ingredients can serve as effective, safe, and inexpensive alternatives for faster wound-healing action. Chemical therapeutics for wound contraction constitute only 1%–3% of Western Pharmacopoeia compared to nearly 30% of plant-based active ingredients, indicating the significant potential of natural products in this context. Moreover, herbal medicine has been utilized as therapeutic alternatives for wound curing because of its increased efficiency and reduced side effects. Plant-based products were extensively used as therapeutic solutions by 65% of the world population and by 80% of people living in developed countries.[7] Therefore, the examination and providing scientific background for plants with ethnopharmacological utilization for skin disorders has been doubled in the last few decades in various distinct geographical areas, the Balkans,[8] China,[9] Iraq,[10] Saudi Arabia,[11] and Iran.[12] In particular, the Flora and ethnobotany studies conducted in nearby regions of Erbil, Iraq (Ballakayati, Shaqlawa, and Rawanduz) and Sulaimani, Iraq (Hawraman and Çuar qurne) have detailed the utilization forms, local names, and medicinal purposes of numerous naturally growing traditional plants. *Cuminum cyminum* (local name, Zeera), as traditional therapeutics has been utilized for health purposes (wound management, Colitis, menstrual irregularities, Hairsutism, Provoking lactation, Carminative) in middle east countries including Iran, Turkey, and Iraq).[13–16]

Cuminum cyminum L. (cumin) is an annual aromatic plant species belonging to *Apiaceae* family. The cumin seeds have a strong taste that has been utilized as a food spice by many nations. Phytochemical analysis revealed several chemicals as main constituents of cumin seeds including γ-terpinene, ρ-cymene, β-pinene, and cuminaldehyde (4-isopropylbenzaldehyde); these compounds were associated with numerous biological potentials.[17,18] Cumin (*Cuminum cyminum*) is well-document as rich source of terpenes, phenolic, and flavonoids.[19] Nonetheless, Umbelliferae family has shown a variety of chemical compounds bearing some important bioactivities.[17] Previously, a detailed phytochemical study revealed the chemical profile of Cumin seeds, which mainly included flavonoids, coumarins, alkaloids, saponins, tannins, vitamins, and minerals.[20] Moreover, organic acids such as propionic, oxalic, aspartic, tartaric, and maleic acids can be detected in the extracts of CCS. These phytochemicals have majorly correlated with their biological potentials, antioxidants,[21] hypoglycemic,[22] anti-inflammatory.[23] A short study on the wound-healing effect of CCS showed the significant potential of these plants

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in accelerating the healing process of incision, excision, and granuloma wounds.[24]

Traditionally, cumin seeds have been ingested as Ayurvedic medicine for many health disorders, including diarrhea, jaundice, dyspepsia, carminative, and as antispasmodic agents.[25] Recently scientists have shown significant efficacy of this plant organ as anti-radical,[26] anti-inflammatory,[23] and anti-diabetic.[22] Ethnobotanical research also reported that people have utilized cumin seeds for wound healing and swollen skin by mixing and melting it with animal fat utile becomes greasy and applies it on injured areas.[13] Here, the present procedure designed to investigate the wound healing actions of *Cuminum cyminum* L. (cumin) in rats by different biochemical and immhunohistochemical techniques.

2 | MATERIAL AND METHODS

2.1 | Plant preparation

Cuminum cyminum L. seeds (CCS) were gathered on Safeen Mountain, Shaqlawa district, Iraq. The plant species were authenticated by Prof. Abdullah Sardar at the College of Education, Salahaddin University-Erbil were air-dried and powered to a coarse powder. The powder transferred into ethanol: water (7:3 ratio) at incubated (60°C) in an ultrasonic bath at room temperature (2 h). The rotary evaporator (40°C) was used to separate the extract solution. The solution moved into a rotary vacuum evaporator to eliminate the solvent. After freeze-drying, the extract was kept in a vacuum desiccator for later analysis.[27]

2.2 | Chemicals

The intrasite gel purchased commercially (Sigma Ald-rich, China), which was used as standard therapeutic for skin injury containing polymer (2.3%), carboxymethyl cellulose (CMC), and propylene (20%). CCS was dissolved by 10% Tween 20 and poured into dark vials for the acute toxicity and wound experiment.

2.3 | Ethic approval

The laboratory handling of animal rats was following the international regulations for animal care set by National scientific instructions.[28] The study protocol approval by the Tishk International University (No. 12, 19/11/2023, M.A.A.).

2.4 | Acute toxicity trial

The toxicity trial was in accordance with standards set by OECD-423.[29] Briefly, thirty-six Sprague Dawley rats (equal number from both genders in 7–8 ages), weighted 180–200 g, were randomly grouped in three cages: A, normal rats without any treatment; B, rats ingested orally 2 g/kg of CCS; C, rats ingested 5 g/kg of CCS. Prior to supplementation, rats were fasted for overnight and after supplementation, water and food were removed for 3–4 h. The procedure of behavioral observation began after 30 min of supplementation procedure and continued for 24 h for any possible sign of toxicity and physical changes (Mild tremors, frightened, eye color, and shortness of breath).[30] The physiological properties including respiration, skin piloerection, salivation, locomotion, and exopthalmus were also compared between normal and CCS-treated rats. On day 15th, rats had an overdose of xylazine and ketamine in 3 mg/kg and 30 mg/kg, respectively, and were sacrificed. Organs were analyzed by histopathological techniques and intercadinal blood samples analyzed for biochemical contents.[31]

2.5 | Wound healing experiment

2.5.1 | Wound excision

Twenty-Four rats (Sprague Dawley) were arbitrary clustered in four cages (6 rats each). For the adaptation purposes, rats were placed in mesh wire cages with full access to tap water and a standard diet (rat pellet) for 7 days. After that, all rats received a small dose of xylazine and ketamine in 12.5 mg/kg, 87.5 mg/kg, respectively, and shaved their dorsal neck and disinfected with 70% alcohol. An equal cut precise cut (2.00 cm, diameter) was created on each rat's dorsal neck by using a round seal[32] (Figure 1). Then, the rats received different treatments on their dorsal neck twice daily:

A, rats had topical treatment of normal saline (0.2 mL).
B, rats had topical intrasite gel therapy (0.2 mL).
C, rats had 0.2 mL of 250 mg/kg of CCS.
D, rats had 0.2 mL of 500 mg/kg of CCS.

The closure area of wounds was estimated by labeling it in square millimeters. The closure area of wounds was determined at different periods (at 0, 5, 10, and 15 days after excision). The wound closure percentage and epithelialized areas were estimated by using a marker, transparency, paper squares, and graph paper (1 mm^2) paper after delivery of partial anesthesia (0.1 mL/20 g) through intraperitoneal injections.[33] The closure percentage calculated as follows:

$$Closure\ \% = [(WA\ on\ day\ 0 - WA\ on\ specific\ day\ X)/(WA\ on\ day\ 0)] \times 100$$

After the estimation of recovered wound area, the experimental rats were given an overdose of 3 mg/kg xylazine and 30 mg/kg ketamine) on day 15 and sacrificed. The skin pieces from recovered wound area were excised and analyzed by different histopathological assays. The blood samples were taken into biochemical evaluations.[34]

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FIGURE 1 Excisional cut on dorsal neck was created in all rats at day 0.

2.5.2 | Histology of wound tissues

The recovered skin tissues were investigated by using different tissue staining techniques. The precise newly generated skin pieces transferred into fixed in 10% phosphate-buffered formalin. The healed tissues transferred into an automated machine for tissue paraffinization, fixation, dehydration, clearing, and infiltration. Sections (5 μm) of skin tissue were fixed on slides followed by a staining procedure utilizing Hematoxylin, Eosin, and Masson's trichrome). After incubation for overnight, the microscopic (Nikon) evaluation of slides performed for any possible histological alterations, deposition of collagen, inflammatory cell infiltration, proliferation of fibroblast, epithelialization, and neovascularization.[32]

2.5.3 | Immunohistochemistry

The present immunohistochemical analysis followed the streptavidin-biotin technique as scientists explained. The immunohistochemical secretion in healed tissues was determined following streptavidin-biotin procedure.[35] Briefly, blocking of endogenous peroxidase with 3% hydrogen peroxide and methanol for 10 min. The mixture was kept for overnight in the incubator (room temperature) in combination with TGF—β (Clone sc-146) diluted antibody 1:50 following the factory's protocols. After slide preparation, slides transferred into incubation (120 min) along with the biotinylated 2nd antibody and streptavidin-peroxidase. The slides were colored with Mayer's hematoxylin as counterstained and examined under microscope.

2.5.4 | Enzyme activity in skin homogenate

The repaired wound tissues were homogenized by utilizing a Teflon homogenizer (Polytron, Germany) at 4°C. The mixture was transferred into centrifuge (4,500 rpm at 4°C) for 15 min. The supernatants were separated for the SOD, CAT, and MDA determination by using ELISA) kits according to instructions mentioned by producer's Company. Kits for evaluating the antioxidant enzymes and MDA content were purchased commercially (Sigma Aldrich, China).[36] For Hydroxyproline evaluation, the skin tissue mixture was centrifuged at 4500 rpm for 15 min at 4°C to obtain the supernatant. The separated supernatant evaluated for the amount of hydroxyproline (HXP) content by using a commercially available ELISA kit, according to the instrument catalog (Sigma Aldrich, China). The entire step-by-step procedure including precision, specificity, optimization, and reproducibility can be found elsewhere.[32]

2.6 | Statistical analysis

The data outcomes are shown as mean ± SEM after triplicate calculations. The statistical evaluation was performed using different methods including one-way analysis (ANOVA, SPSS software) and Graph Pad Prism 9.0. Values were found significant at $P < 0.05$.

3 | RESULTS

3.1 | Acute toxicity

The current study revealed the safety of a methanolic extract of CCS ingestion (2 and 5 g/kg) for 14-day. The observation of rats every 8 h has not detected any abnormal behavior or physiological alteration in rats. The present oral supplementation of CCS did result in any toxic signs (restlessness, pain, wound area bite, convulsion, and irritation) in rats. CCS-treated rats functioned very normally throughout the procedure and they ingested an equal amount of feed intake and body weight compared to that of normal controls. The dissected liver and kidney organs from supplemented and normal rats had similar histological profiles of their tissues as seen in Figure 2. The biochemical evaluation of serum specimens revealed non-significant changes vehicle and CCS-treated rats (Available on request).

3.2 | Wound experiment

3.2.1 | Wound size and closure percentage

The present study found a significant healing effect of CCS in both utilized doses 250 and 500 mg/kg as shown in Table 1. As illustrated in

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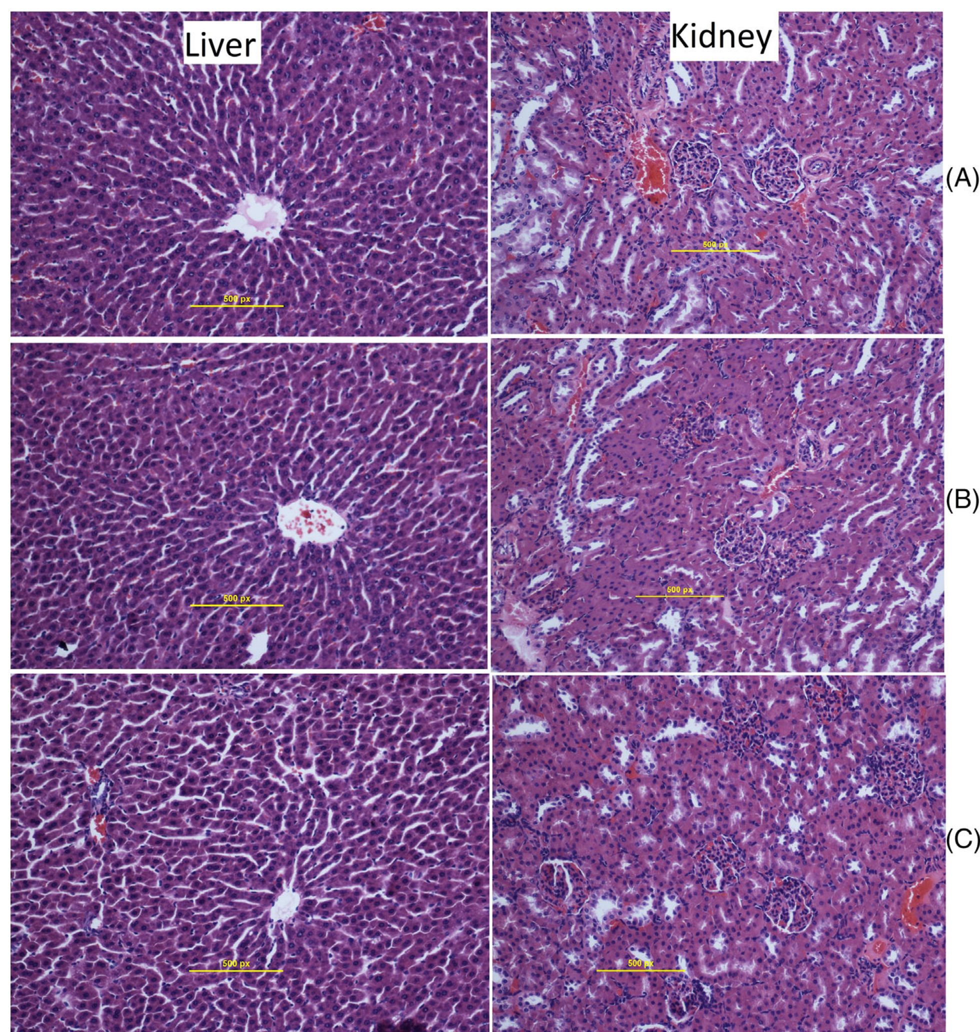

FIGURE 2 Effect of methanolic extract of CCS on rats in acute toxicity trial. (A) Vehicle rats; (B) low (2 g/kg) dose of CCS extract; (C) high (5 g/kg) dose of CCS extract.

TABLE 1 Effect of CCS on the percentage (%) cured wounds in rats.

Clusters	Wound area (mm^2) Day 0	Wound area (mm^2) Day 5	Day 5 closure (%)	Wound area (mm^2) Day 10	Day 10 closure (%)	Wound area (mm^2) Day 15	Day 15 closure (%)
A	240 ± 0.56	158.34 ± 2.1[c]	34	88.43 ± 0.34	63.16[b]	44.21 ± 0.9	81.57[b]
B	240 ± 0.46	88.20 ± 1.43[a]	63.25	33.67 ± 1.3[a]	85.97[a]	12.0 ± 1.0*	95[a]
C	240 ± 0.44	95.2 ± 1.20[b]	60.33	46.32 ± 1.2[b]	80.7[a]	21.2 ± 0.8*	91.16[a]
D	240 ± 0.66	93.54 ± 1.14[b]	61.02	41.0 ± 1.2[b]	82.91[a]	15.9 ± 0.78*	93.37[a]

[a]Rats had normal saline.
[b]Rats had topical treatment of intrasite gel (reference).
[c,d]Rats had topical treatment of 250 and 500 mg/kg of CCS. Similar superscript on values within same column indicates non-significant at $P < 0.05$.

Figures 3, 4, and 5, different rates of skin recovery were found between groups, however, only intrasite gel or CCS treatments caused significant wound healing effects after 5, 10, and 15 days of application. Rats treated with intrasite gel had significant curing signs and their skin recovered more rapidly than that of gum acacia (vehicle) or CCS-treated groups throughout the procedure. CCS treatment (500 mg/kg) had less wound area after 5, 10, and 15 days of trial in comparison to normal saline-treated rats (Figures 3, 4, and 5). After 14 days of

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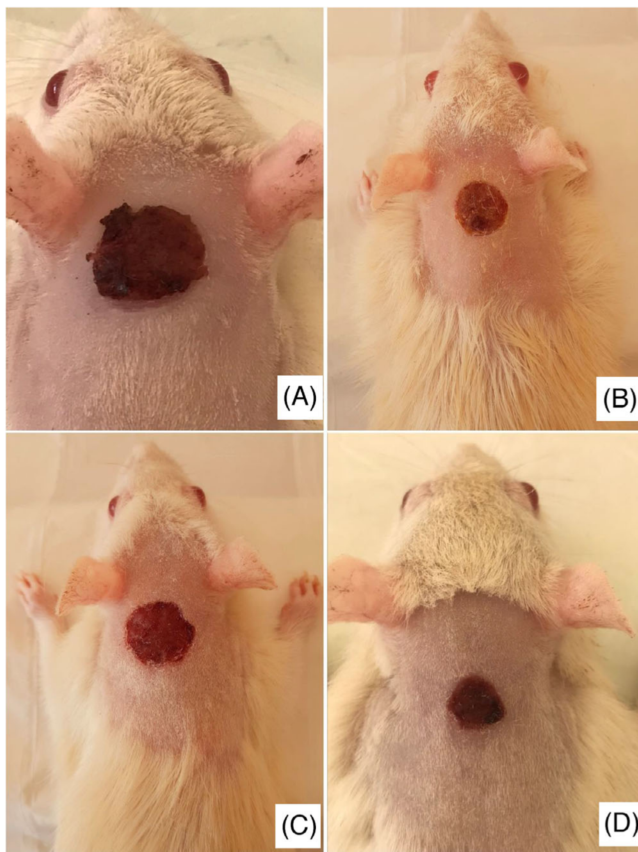

FIGURE 3 The dorsal neck injury on day five after excision. (A) Vehicle rats had 0.2 mL of normal saline; (B) rats treated with 0.2 mL of intrasite gel had smaller wound size compared to other groups; (C and D) Rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS shows smaller wound size (mm^2) and higher percentages of wound contraction in compare normal saline-treated rats.

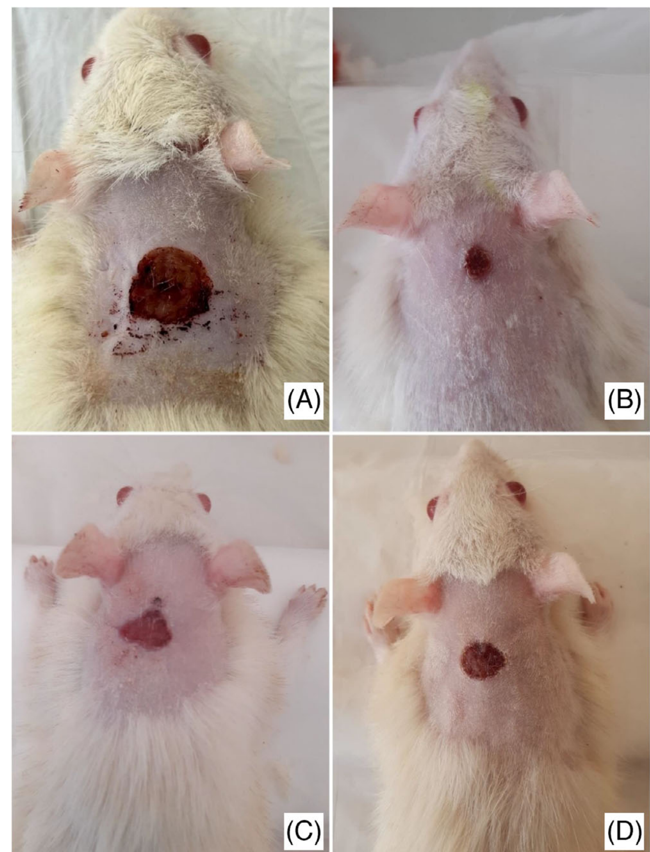

FIGURE 4 The dorsal neck injury on day 10 after excision. (A) Vehicle rats had 0.2 mL of normal saline; (B) rats treated with 0.2 mL of intrasite gel had smaller wound size compared to other groups; (C and D) rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS shows smaller wound size (mm^2) and higher percentages of wound contraction in compare normal saline-treated rats.

applications, the closer percentages of wounds were remarkably higher (93.37%) in rats received 500 mg/kg of CCS than that (81.57%) of vehicle rats, but the values were very much comparable to that (95% and 91.16%) of intrasite gel or 250 mg/kg CCS-treated rats, respectively (Table 1 and Figure 5).

Gross morphology results showed that the percentage of cured wounds in the gum acacia-treated rats (A) was significantly lower during all periods of the procedure compared to that of CCS and intrasite gel-treated rats (Figures 3, 4 and 5). Rats addressed topically with 0.2 mL of 250 and 500 mg/kg CCS had faster wound skin repair during all periods of measurement, which were gradual improvement based on time. On days 10 and 15 after skin excision, rats received CCS (0.2 mL of 500 mg/kg) had similarly repaired wound size in comparison to intrasite gel-treated rats (Figures 4 and 5). After 15 days of wound treatment, rats treated with 0.2 mL of 500 mg/kg CCS had significantly smaller wound areas and higher percentage of wound contraction in comparison to normal saline-treated rats and almost same as values for intrasite gel-treated rats (Table 1 and Figures 4 and 5).

3.2.2 | Histopathological effect of CCS on wound tissues

The repaired skin tissues were analyzed by different histopathological assays using different stains (H & E and Masson Trichrome). The normal saline-addressed rats (group A) had significantly decreased tissue granulation based on the microscopic observation of skin tissues stained by H & E. In comparison, wound tissues treated with intrasite gel (group B) or 0.2 mL of 250 (group C) and 500 mg/kg CCS (group D) showed higher repaired wounds, more collagen formation, more vascularization, and fewer inflammatory cells in their granulated tissues compared to vehicle rats (Figures 6 and 7). The repaired wound tissues from different treated rats showed various levels of dermal growth and collagen deposition based on Masson's trichrome technique. Rats who received topical application of normal saline had collagen rearrangement and significantly less regeneration of skin tissue in comparison to treated rats. Moreover, vehicle rats had increased inflammatory cells, important mediators of wound healing, which were dispersed all over the wound area with decreased granulated cells (immature skin),

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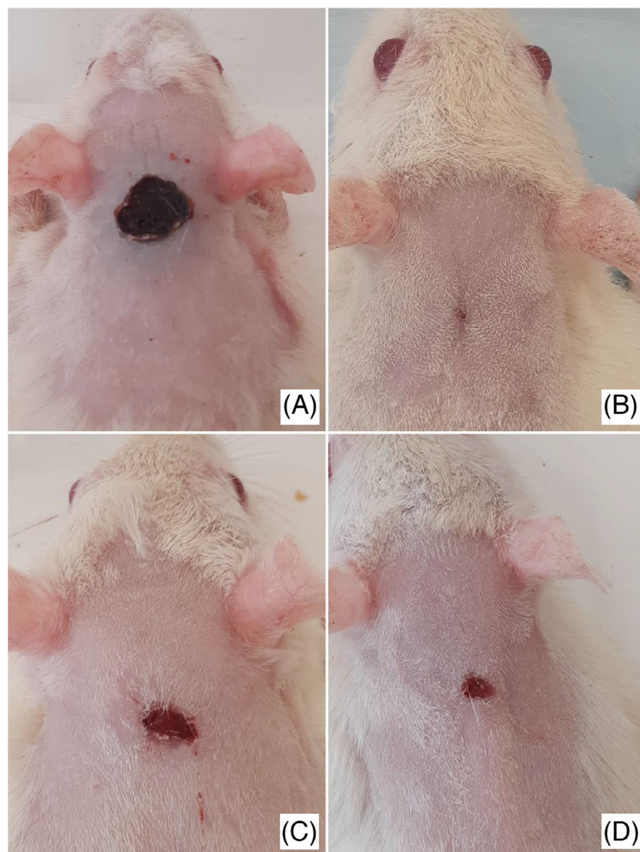

FIGURE 5 The dorsal neck injury on day 15th after excision. (A) Vehicle rats had 0.2 mL of normal saline; (B) rats treated with 0.2 mL of intrasite gel had smaller wound size compared to other groups; (C and D) rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS shows smaller wound size (mm^2) and higher percentages of wound contraction in compare normal saline-treated rats. The estimated values of wound closure of 0.2 mL of 500 mg/kg CCS-treated rats were similar to intrasite gel-treated rats.

indicating less skin tissue regeneration consequently delays skin recovery. While CCS-treated rats had increased production and collage fiber deposition in a better-arranging format than that of normal rats, but very comparable to intrasite gel group (Figures 8 and 9).

3.2.3 | Effects of CCS on TGF-β1 expression

The histopathological evaluation of the recovered skin tissues after 15 days of different treatments showed significantly various intensity of TGF-B1, denoting an extensive range of tissue remodeling and autoimmune transformation. Normal saline-treated rats expressed the lowest level of TGF- β1 expression compared to that of all other treated rats. While rats received 0.2 mL of 250 and 500 mg/kg of CCS showed elevated TGF-β1 expression in their recovered skin, which was more abundant than in the vehicle rats, but much comparable to that of intrasite gel-treated rats (Figure 10). The outcome indicates increased provoking effect of CCS on the growth and myofibroblast formation (facilitating angiogenesis and skin tissue repair).

TABLE 2 CCS effects on antioxidants and MDA levels in tissue homogenates.

Animal groups	SOD (U/mg protein)	CAT (μmmol/min/ mg protein)	MDA (nmol/mg protein
A	3.36 ± 0.20^c	22.83 ± 1.49^c	7.63 ± 0.16^c
B	8.033 ± 0.29^a	45.16 ± 1.72^a	2.47 ± 0.16^a
C	5.68 ± 0.11^b	37.83 ± 1.16^b	3.58 ± 0.17^b
D	7.05 ± 0.258^a	39.66 ± 2.73^b	2.73 ± 0.12^a

[a] Rats had normal saline.
[b] Rats had topical treatment of intrasite gel (reference).
[c,d] Rats had topical treatment of 250 and 500 mg/kg of CCS. Similar superscript on values within same column indicate non-significant at $P < 0.05$.

3.2.4 | Effect of CCS on antioxidants and MDA

The endogenous antioxidants in wound tissue homogenates were found in different concentrations, which were significantly different between vehicle rats compared to treated rats. Rats who received gum acacia (vehicle) had the lowest tissue antioxidant enzymes (SOD and CAT), indicating severe oxidative stress due to increased ROS formation. However, rats treated with intrasite gel or CCS showed increased levels of tissue antioxidants than in the vehicle rats. CCS treatment (500 mg/kg) caused significant up-regulation of SOD (7.05 U/mg) and CAT (39.66 μm mol/min/mg), which were more than that (3.36 U/mg and 22.83 μm mol/min/mg, respectively) of normal saline-treated rats. The lipid peroxidation level (MDA) was found significantly elevated in gum acacia-treated rats, causing further oxidative stress-related damage and delaying the wound healing process. While, rats who received topical treatment of intrasite gel or 500 mg/kg CCS showed reduced MDA (2.47, 2.73 nmol/mg, respectively) levels in their wound tissues than 7.63 nmol/mg in the vehicle rats. The outcome suggests noticeable antioxidant potentials and ameliorative actions of CCS against lipid peroxidation in skin tissues in rats (Table 2).

3.2.5 | CCS effects on hydroxyprolyine

The present results showed a statistically different level of hydroxyproline (major amino acid of collagen) in recovered skin tissues obtained from experimental rats (Figure 11A-D). Vehicle (gum acacia) rats showed the lowest concentration (34.5 mg/g) of hydroxyproline, indicating reduced collagen fibers in regenerated tissues. The intrasite gel-treated rats had a significant up-regulation (82.5 mg/g) of hydroxyproline, which was expected because of provoking action on cellular proliferation and collagen formation compared to reduced collagens in vehicle rats. Rats treated with 0.2 mL of 500 mg/kg CCS (Figure 11) showed very comparable values to that of reference rats and significantly higher levels (71.65 mg/g) of hydroxyproline than (34.5 mg/g) in the vehicle rats, indicating more collagen fibers, thus faster wound healing actions. Data analysis also found a non-significant difference

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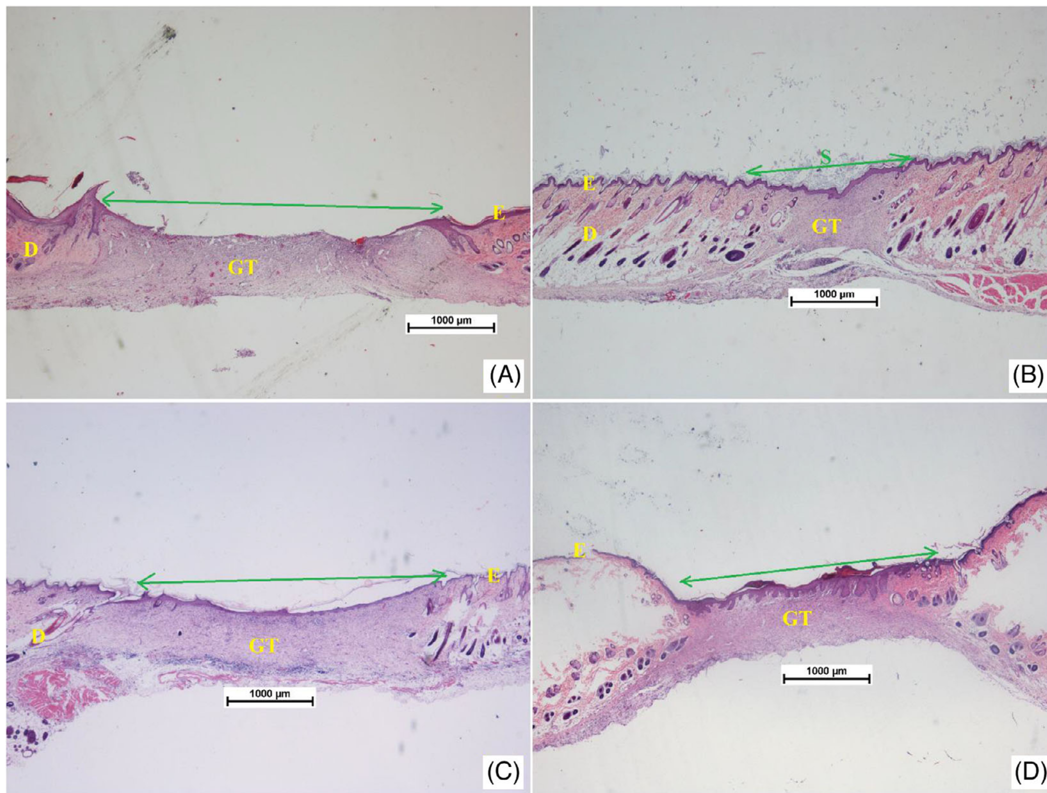

FIGURE 6 Microscopic appearance (high magnification) of injured skin tissues on day 15 after surgery. (A) Rats had 0.2 mL of normal saline revealed increased wound area; (B) rats had 0.2 mL of intrasite gel revealed smaller wound size compared to other groups; (C and D) rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS had moderate to small wound area in compare to normal saline-treated rats. Green arrow, closure area of wound closure; S, scab; GT, granulation tissue; E, epidermis (H&E stains, 2×).

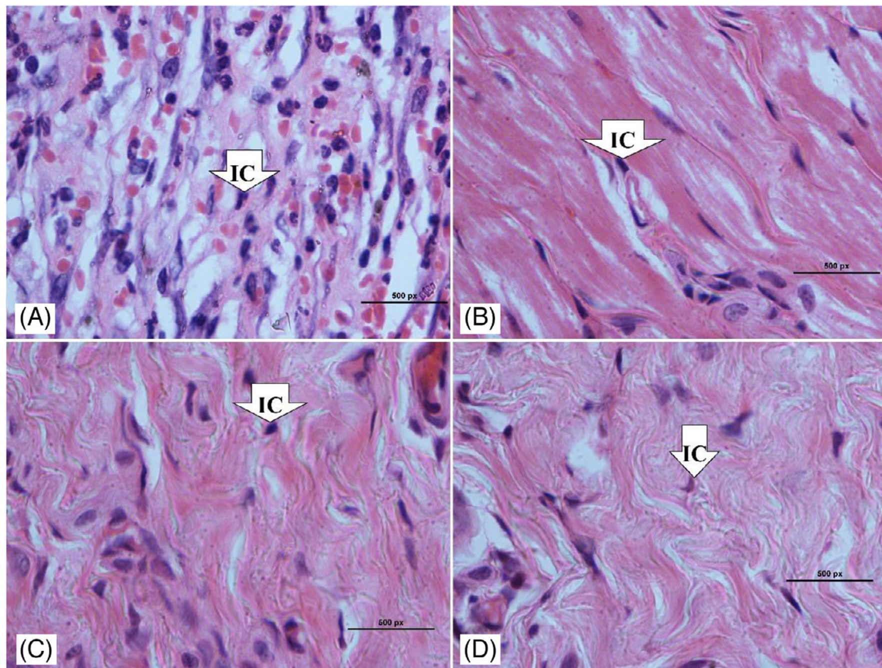

FIGURE 7 Microscopic appearance (high magnification) of injured skin tissues on day 15 after surgery. (A) Rats had 0.2 mL of normal saline increased inflammatory cells (IC) and reduced fibroblast infiltration ; (B) rats had 0.2 mL of intrasite gel revealed smaller wound size compared to other groups; (C and D) rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS had moderate to small wound area and reduced inflammatory cells in compare to normal saline-treated rats (H&E stains, 100×).

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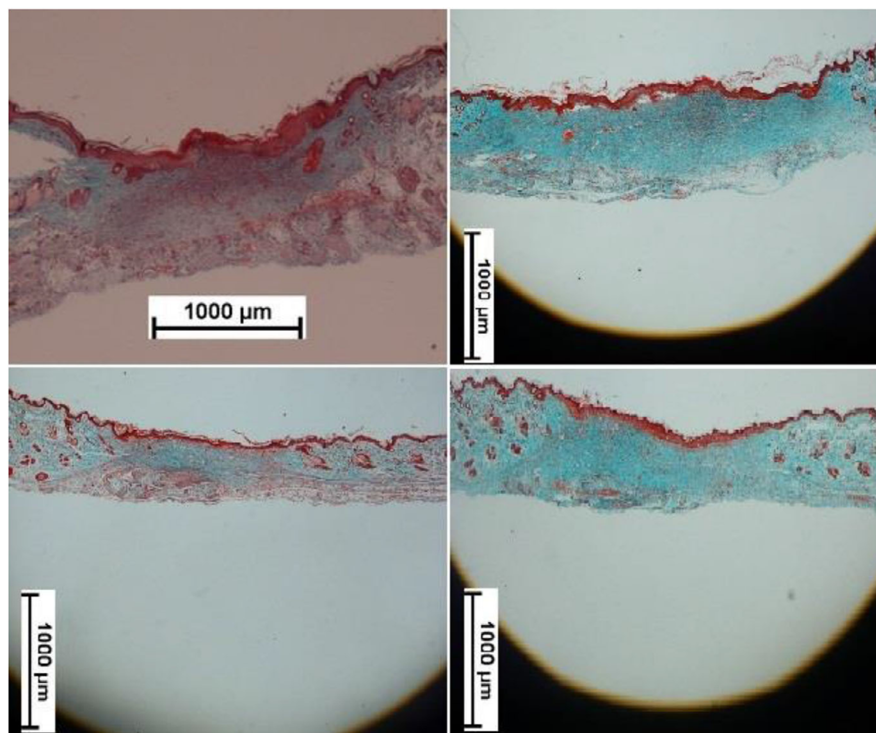

FIGURE 8 Microscopic observation of injured tissues stained on day 15 after excision in rats stained by Masson's Trichrome; (A) rats had 0.2 mL of normal saline shows highest wound size and reduced fibroblast, immaturity of the dermis, and reduced collagen deposition in their granulation tissue (GT). (B) rats treated topically with 0.2 mL of intrasite gel (100 mg/mL) showed significantly the lowest wound area and increased collagen deposition (deep green color). (C) rats received topically 0.2 mL of 250 mg/kg of CCS showed smaller wound area, moderate collagen content (moderate green color), and significantly increased dermal growth. (D) Rats addressed with 0.2 mL of 500 mg/kg of CCS showed increased wound closure, elevated fibroblast, more dermal maturation, and higher collagen content (green) in their GT. (Magnification 2×).

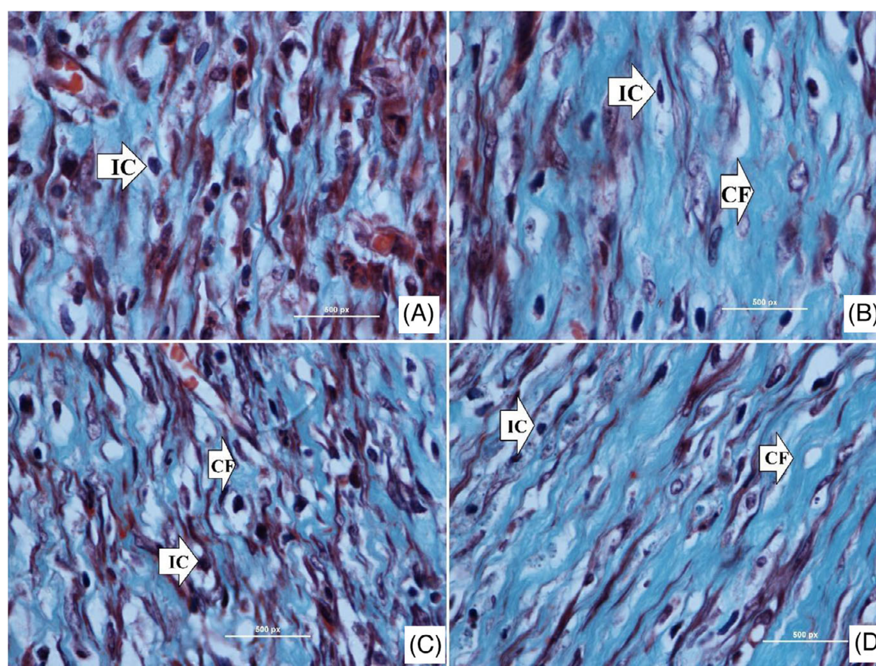

FIGURE 9 Microscopic appearance (High magnification) of injured skin tissues on day 15 after surgery (Masson's trichrome stain). (A) Vehicle rats had 0.2 mL of normal saline revealed increased wound area, increased inflammatory cells (arrows), less collage fiber, and fewer fibroblasts; (B) rats had 0.2 mL of intrasite gel revealed smaller wound size compared to other groups, had noticeably reduced wound area and less collagen deposition (deep green color); (C and D) rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS had moderate to small wound area in compare to normal saline-treated rats. CCS-treated rats showed significantly increased tissue collagen deposition (moderate green color) and fibroblasts proliferation, and less inflammatory cells (Magnification 100×).

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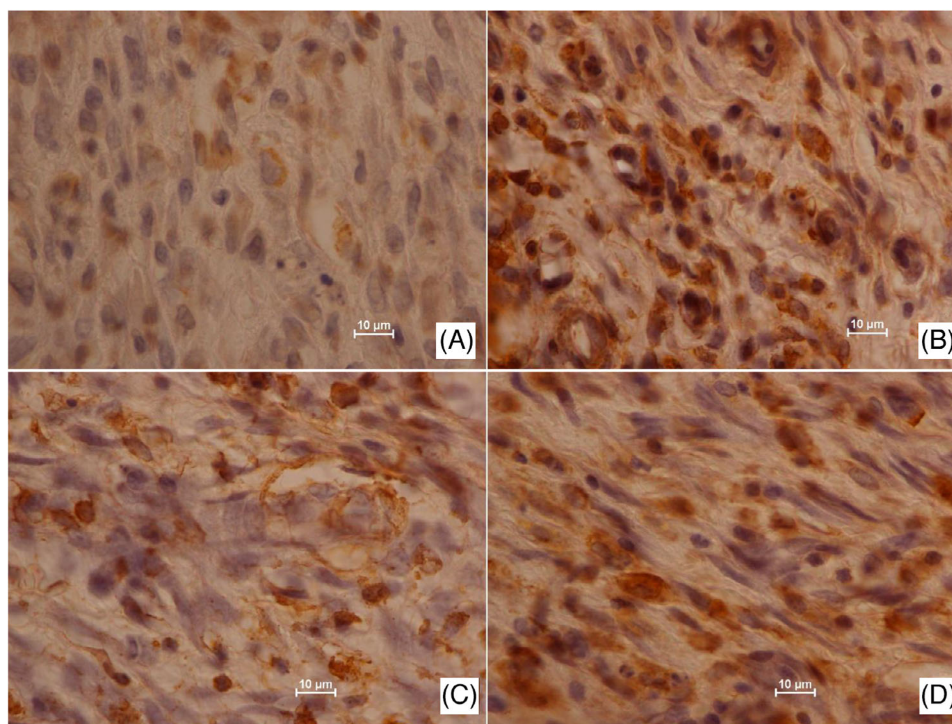

FIGURE 10 Microscopic appearance (High magnification) of injured skin tissues expressing different TGF- β1 intensity on day 15 after excision. (A) Vehicle rats had 0.2 mL of normal saline revealed reduced expression of TGF- β1 represented by very light brown stain; (B) rats had 0.2 mL of intrasite gel had highest TGF- β1 expression (intense brown color); (C and D) rats treated topically with 0.2 mL of 250 and 500 mg/kg of CCS had moderate to small wound area in compare to normal saline-treated rats. CCS-treated rats showed significantly increased expression of TGF- β1 (intense brown color tissues), denoting higher tissues regeneration in healed skin area in compare to normal saline-treated rats (Magnification 100×).

between rats that received 0.2 mL of 250 mg/kg CCS and positive control (vehicle) rats.

The pharmaceutical industry still lacks a novel therapeutic capable of accelerating the curing process of skin injury without any side effects. The available skin pharmaceuticals applied for wound healing comprise only 1%–3% of the medicines mentioned in Western pharmacopoeias; by comparison, the same percentage of medicinal plants have been utilized for the same purpose.[37] Ethnopharmacological investigation on traditional medicinal plants may discover new and active ingredients with therapeutic potentials of wound healing.[13]

The toxic effect of medicinal plants and their natural ingredients is considered one of the main drawbacks associated with utilizing them as a treatment for human disorders.[38] In the present work, the acute toxicity test of methanolic extracts (2 and 5 g/kg) of CCS revealed the absence of any noticeable change in the appurtenance or physiology of rats without any mortality record after two weeks of supplementation. Moreover, histopathological and biochemical evaluations revealed comparable results between normal control and CCS-treated rats. Accordingly, researchers reported the safety of ingesting 2 g/kg of a methanolic extract of CCS to mice with zero mortality record even after the experimental period.[39] Similarly, numerous research data have been published declaring non-significant toxic effects of ingesting (2-5 g/kg) CCS in rats.[22,40,41]

The provoking action in wound healing process is commonly evaluated by two techniques, incisional and excisional. The present study applied a full-thickness excisional wound procedure to evaluate the wound-curing efficacy of CCS. The vehicle group showed common stiff wounds with dark brown scabs, characterized by the largest wound area, early epithelialization, and reduced closure percentages. While a topical application of CCS on excisional wounds promoted wound contraction and epithelialization represented by a higher wound closure percentage compared to the vehicle group. The epidermal layers in CCS-treated rats produced around the wound area were thicker than that of the vehicle group, thereby preventing further tissue damage by coating the area around the wounded skin.[34] The histological profile of wounded tissues from CCS-treated rats demonstrated a well-organized granulation of tissues and newly vascularization and significantly less inflammatory mediators than in the untreated vehicle group. The microscopic observation of regenerated tissues, colored by H&E and Mason Trichoma stains, revealed noticeable tissue regeneration enhanced by the topical application of CCS, which was by the previous outcomes of wound-curing effects of CCS extracts.[24] Similarly, researchers have shown the wound-healing potentials of CCS obtained from a variety of medicinal plants.[42–44]

The TGF-β1 is a well-known multifunctional polypeptide factor considered an important mediator of tissue regeneration (angiogenesis) and faster wound repair because of its modulatory action on

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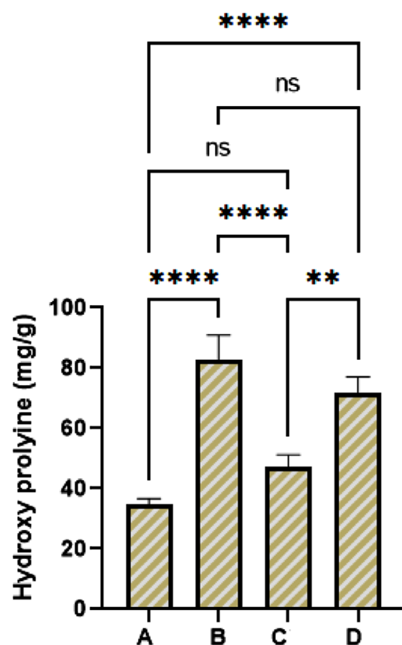

FIGURE 11 Effect of CCS on the hydroxyproline content in homogenate skin. (A) Rats had normal saline; (B) rats had topical treatment of intrasite gel (reference); (C and D) rats had topical treatment of 250 and 500 mg/kg of CCS. There were significantly higher HXP levels in skin tissues of intrasite gel and CCS-treated rats, aiding in higher collagen formation and a faster wound closure process.

the ECM proliferation and regeneration by modulating the mesenchymal cell growth.[45] Recent decades witnessed tremendous research data explaining the molecular mechanism of TGFβ, which is regulated by many receptor-ligand interactions and intracellular mechanisms.[46] Previous findings showed that TGFβ1 cytokine can have an inhibitory effect on cellular proliferation (epithelial cells), on the other hand, it may induce proliferation process and ECM deposition in other cells (mesenchymal cells).[47] In case of wound healing stress, synthesis and release of TGFβ1 proteins increased dramatically enhance the release of inflammatory mediators thereby aiding in faster wound contraction.[35] Therefore, any modulation in the expression and mechanisms of TGFβ1 protein can have different effects on wound contraction. As the level of these proteins increases in the wound bed, TGFβ1 can be considered a major contributor to cell survival and tissue regeneration within curing wounds.[48]

In our study, the immunohistochemical analysis revealed that untreated vehicle rats expressed less TGFB1 intensity in their wound tissue sections represented by a very light brown color. Contrarily, rats that received topically CCS (250 and 500 mg/kg) showed elevated TGFB1 expression in their sectioned wound tissues shown by intense brown color, very comparable to intrasite gel-treated rats. The outcomes denote the efficacy of CCS in the up-regulation of TGFB1 proteins in wounded skin which promoted the wound-curing process. Accordingly, researchers revealed the efficacy of CCS extracts in the augmentation of immunohistochemical proteins.[41] Moreover, supplementation (200 mg/kg) of CCS to a hypertensive rat model in a nine-week experimental trial caused significant augmentation of immunohistochemical (Bax/Bcl-2) proteins, which are apoptotic proteins capable of regulating the oxidative stress and various inflammatory process.[49]

The wound repair accompanied with skin ischemia, which promotes the initiation of ROS by activated leukocytes in the wound area. As the action progresses, the increased free radicals' formation in the wound tissues stimulate inflammatory cytokine release which increases leukocyte infiltration (chemotaxis) and higher oxidative damage in these tissues. To prevent such consequences, the body produces antioxidant enzymes that will balance the produces free radicals in the injured tissues. However, in case of disease, antioxidants cannot fightback the tremendous reactive oxygen species which will consequently disrupt cellular structure and function leading to delayed wound recovery. Cells can have different antioxidant enzymes as their defense mechanism to eliminate the free radicals and prevent oxidative stress-related damage during stress conditions. The antioxidant enzymes (CAT, SOD, and GPx) comprise a major part of the antioxidant defense system of cells that can effectively ameliorated oxidative injury, thereby avoiding disorders associated with increased reactive oxygen species.[50] The SOD is major antioxidant that reduces risks of oxidative damage by the dismutation of O2- to H_2O_2 and O_2. The catalase (CAT) enzyme is a well-known antioxidant that suppresses oxidative stress by changing H_2O_2 to H_2O and oxygen. Because overcrowding of H_2O_2 in cells can initiate a Fenton reaction, which generates more reactive oxygen reactive resulting in severe oxidative-related cellular damage. The methanolic extracts of CCS showed significant radical quenching actions represented by lower SOD and CAT levels in rat's tissue homogenate, thereby reducing the rate of oxidative damage which can have a devastating effect on wounded tissues and the wound process of wound curing. Accordingly, numerous investigators reported the antioxidant efficacy of CCS extracts in separate trials (in vivo and in vitro), which were linked with their phytochemical profiles (terpenes, phenolic, and flavonoids).[19,51,52]

The elevation of free radicles and increased rate of oxidative stress can generate a process called lipid peroxidation, which is a series of lipid degradation processes that can have a destructive effect on the cell organelles and can increase membrane permeability, altering collagen metabolism, proliferation of fibroblast, and easy access of keratinocyte. The lipid peroxidation in damaged skin tissues near can have inhibitory action on the production of endothelial growth factor (vascularization), causing further delay in wound contraction. MDA is considered as reactive molecule and a precise indicator of lipid peroxidation that result from oxidation of polyunsaturated fatty acids. The present data showed elevated MDA levels of MDA in homogenate tissues from normal saline-treated rats. While rats receiving intrasite gel or methanolic extracts of CCS had significantly reduced values of MDA, denoting antioxidant potentials of these agents against oxidative damages that would otherwise delay the wound healing process. Accordingly, studies have shown that ingestion of CCS extracts caused a significant reduction of lipid peroxidation (lower MDA levels) in animal models had chemical-mediated oxidative stress.[52–54]

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A healing tissue in repaired wounds produces increased collagen protein, as one of the essentials required for tissue regeneration and for the final two stages of wound repair. 4-Hydroxyproline (HXP) is the main amino acid of collagen that comprises nearly 13.5% of its amino acid content which allows collagen twisting helix to maintain its integrity and structural stability.[55] Researchers have repeatedly confirmed that the faster the wound contraction can be associated with the higher the HXP formation. In the case of our study, vehicle rats had the lowest level of HXP because they did not receive any treatments. While, CCS-treated rats had significantly elevated HXP values in their wound tissue homogenates, denoting higher collagen content that why they had higher wound closure percentages. This outcome was supported by the previous reports, which indicated the phytochemical contents (*p*-mentha-1,4,-dien-7-al, cumin aldehyde, and terpenoid) of CCS that could be associated with its stimulatory effects on the collagen synthesis process in the wound tissues.[56,57]

4 | CONCLUSION

The present investigation aimed to appraise the wound recovery potentials of methanolic extracts of CCS in rats. The toxicity evaluation of CCS showed absence of abnormal alteration, physiological change or even death in rats ingested with methanolic extract of CCS. CCS treatment caused significant provoking of wound contraction and noticeably increased the wound closure percentages, more efficiently at 500 mg/kg based on histological (H&E and Mason Trichoma), immunohistochemical, biochemical, and hydroxyproline evaluations of recovered wound tissues. The present study's limitation includes animal housing shortage and low budget. Therefore, future studies are required to analyze the molecular aspects of CCS associated with the mechanism of wound healing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

The laboratory handling of animal rats was following the international regulations for animal care set by National scientific instructions.[58] The study protocol approved by ethical committee of Tishk international University (No. 2, 11/12/2023/M.A.A.)

DATA AVAILABILITY STATEMENT

Details regarding the current will be available on request from the corresponding author (Ahmed A.j. Jabbar).

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