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ORIGINAL ARTICLE

Mechanisms underlying the wound healing and tissue regeneration properties of *Chenopodium album*

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Abstract

In the current study, aerial parts (leaves, stem and shoots) of *C. album* were extracted with methanol and subjected to phytochemical and HPLC analysis. Agar well diffusion method was used for anti-bacterial activity against Gram-negative strains *Escherichia coli*, *Salmonella typhi*, *Klebsiella*, *Pseudomonas aeruginosa* and Gram-positive *Bacillus cereus*, *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus*. Burn was induced through flame heated metal rod on mice. *C. album* ointment (2% w/w), Vaseline (vehicle) and silver sulfadiazine (standard) were topically applied thrice daily for 15 days. Wound area was measured on day 0, 5, 10 and 15. On last day, the wound tissues were excised and subjected to histopathological, quantitative PCR and immunohistochemical analysis. Phenols, alkaloids, phytosterols, tannins, saponins, flavonoids, carbohydrates and glycosides were detected in phytochemical analysis. HPLC chromatogram displayed peaks for rutin, quercetin, ascorbic acid, gallic acid and various other phyto-constituents. The extract exhibited zone of inhibition in millimeter (mm) against *E. coli* (12.3 ± 0.57), S. *typhyi* (14.6 ± 1.52), *Klebsiella* (11.8 ± 0.76), *P. aeruginosa* (12.3 ± 0.57), *B. cereus* (12.5 ± 1.29), *S. aureus* (18.3 ± 2.08), and MRSA (11.8 ± 0.76). The wound area in *C. album* group was significantly (60%) reduced as compared to vehicle group (11%). Histological analysis showed complete re-epithelialization and fine tissue in extract treated group. qPCR data revealed up-regulation of *EGF, PDGF* and *TGF-β1* genes in extract treated group. Similarly, immunohistochemistry results confirmed heightened EGFR expression in extract treated group. Our findings suggest that *C. album* can promote wound healing and tissue regeneration through control of burns related infection and modulation of growth factors and its receptors.

Keywords *Chenopodium album* · Burn wound healing · *EGF* · *FGF* · *PDGF*

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Introduction

Chenopodium album Linn. commonly known as "Lambs quarter" and "Bathu" in English and Hindi, respectively. It belongs to a flowering family, called Chenopodiaceae (Arora and Itankar 2018). It is an edible annual shrub widely grown in Pakistan, India, Africa, North America and Europe (Arora and Itankar 2018). *C. album* is traditionally used for the treatment of burns, high blood pressure, abdominal pain, intestinal ulcers, spleen enlargement, hepatic disorders, eye infections, piles, diarrhea, liver disorders and edema (Poonia and Upadhayay 2015). *C. album* contains quercetin, rutin, chenoalbuside, chenoalbicin, cinnamic acid amides, calenduloside E, chikusetsusaponin, saponins, tannins, steroids, glycosides and terpenoids (Poonia and Upadhayay 2015). Antiproliferative, anti-fungal, anti-viral, anti-inflammatory,

anti-septic, immunomodulatory, giardicidal, nematicidal, spasmolytic, anti-allergic and hepatoprotective activities have been reported for *C. album* and related species of *Chenopodium* genus (Morteza-Semnani 2015).

Burn wounds are the most traumatic and physically devitalizing injuries affecting normal functionality of organs and predisposes the body to pathogen invasion. Untreated or improperly treated wounds lead to significant morbidities and mortalities (Wang et al. 2018). Globally, 300,000 annual deaths are related to burn injuries, in which 90% are from low- and middle-income countries (Sajjad et al. 2019). After burn injuries, natural repair process is initiated, which consists of hemostasis, inflammation, proliferation and remodeling. However, protein-rich avascular microenvironment of wound bed favor invasion and proliferation of microbes, which interfere with healing process. The pathogens that primarily colonize the burn wound are *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella* species of Gram-negative bacteria, and notably methicillin-resistant *Staphylococcus aureus* (MRSA) from Gram-positive bacteria. Emergence of antibiotic resistance among pathogens also limits the therapeutic possibilities for effective treatment of burn wound infections (Halstead et al. 2018). The standard burn wound treatments like grafting and application of ointments are still associated with pain, infection, slow wound healing and hypertrophic scarring (Wang et al. 2018). Similarly, synthetic products cause allergy, drug resistance and toxicity. Therefore, researchers are looking for alternative therapies that may have inherent antimicrobial and healing properties with least adverse effects (Takzaree et al. 2016).

Natural products especially of plants origin may find potential applications in burn wound management. The bioactive secondary metabolites in plants give beneficial properties like anti-inflammatory, anti-septic, anti-microbial and astringent activities (Solanki et al. 2017). These properties may help to control infection and provide optimum healing environment. Therefore, plant based products can offer new avenues in skin regeneration due to cultural acceptability, efficacy, higher safety profile, biocompatibility and cost-effectiveness (Khomarlou et al. 2017). Several studies have reported that burn centers in South America, India and China use crude extract of medicinal plants for the treatment of burn wounds (Das 2013). Still many of the plant species are unexplored, thus, rigorous research is needed to evaluate the potential of medicinal plants for the restoration of normal skin structures with effective control of infection.

C. album, regardless being the medicinally important plant, there is not enough scientific evidence for its use in wound healing and tissue regeneration. Therefore, this study was designed to evaluate the potential of *C. album* for the control of infection, burn wound healing and tissue regeneration. Findings of this study suggest that *C. album* has potential to control infection and promote proper curing of burn wounds through modulation of healing and tissue regeneration related molecular players.

Methods

Chemicals and standard drugs

Methanol, formaldehyde, dimethyl sulfoxide (DMSO), nutrient broth and agar media were purchased from Merck (Darmstadt, Germany). Sodium hydrogen phosphate monobasic (NaH_2PO_4), paraffin wax and haematoxylin were from Sigma-Aldrich (St. Louis, USA). Double distilled water, petroleum jelly (Vaseline), lignocaine HCl (Barrett Hodgson Pakistan), xylazine HCl (Farvet Pan America), ketamine HCl BP (Indus Pharma, Pakistan) and silver sulfadiazine (Ferozsons Laboratories Ltd. Pakistan) were acquired from the local market.

Plant material

The leaves of *C. album* were collected from Abbottabad, Pakistan in the month of July, 2017. The plant material was identified by Dr. Arshad Mehmood Abbasi, Assistant Professor, Department of Environmental Sciences, COMSATS University Islamabad (CUI), Abbottabad Campus, Pakistan. The voucher specimen (No.Ca-A-03/14) was deposited at the herbarium of the respective institute.

Processing and extraction

The collected leaves of *C. album* were washed thoroughly with tap water and finally with double distilled water to remove impurities. The plant material was shade dried and then ground into fine powder. Then, 1 kg of the plant powder was macerated with 2 L of 70% methanol with periodic stirring for 15 days at room temperature. The resulting mixture was first filtered through muslin cloth and then passed through Whatman No. 42 filter paper. The filtrate was concentrated at 40 °C under reduced pressure in vacuum rotary evaporator (Buchi Switzerland rotavapor R-300 system) to obtain a semi-solid extract.

Phytochemical analysis

Preliminary phytochemical analysis of *C. album* extract was carried out to examine phenols, alkaloids, phytosterols, tannins, saponins, flavonoids, carbohydrates, glycosides and amino acids, as per standard protocols (Khan et al. 2016) with some modifications.

The *C. album* extract was further screened for the presence of various phytochemicals through high performance liquid chromatography (HPLC; SHIMADZU, Japan). The

HPLC system was consisted of SHIMADZU LC-20AP pump, coupled with multiwavelength UV–Vis detector (SPD-20A/20AV). For separation, Shim-Pack, C18 analytical column (150 mm × 4.6 mm i.d. × 5 µm) was used at ambient temprature. The mobile phase consisted of methanol and water (90:10% v/v), which was degassed prior to injection into HPLC system. The flow rate was 1 mL/min with an injection volume of 20 µL and UV detection was carried out at 205 and 254 nm. The *C. album* extract was disolved in HPLC grade methanol (10 mg/20 mL) and passed through silica gel. The filtrate was passed through 0.45 µm membrane filter.

Anti-bacterial assay

Anti-bacterial activity of *C. album* extract was evaluated against various burn associated pathogens through agar well diffusion method (Irshad et al. 2012). The selected strains are predominant wound invaders, which include, *Escherichia coli* (*E. coli*), *Bacillus cereus* (*B. cereus*), *Salmonella typhyi* (*S. Typhi*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella, Staphylococcus aureus* (*S. aureus*) and Methicillin-resistant *Staphylococcus aureus* (MRSA). The selected strains were kindly provided by Dr. Ihsan-Ul-Haq, Department of Pharmacy, Quaid-i-Azam University, Islamabad, Pakistan. The pathogenic strains were re-activated by successive culturing on nutrient agar plates. Healthy cultures of microorganisms were inoculated in 100 mL of nutrient broth and incubated at 37 °C for 16–24 h, separately. For the assay, 200 µL of inoculum was spread uniformly on the Mueller–Hinton agar plate. Afterwards, the required number of wells were punched and filled either with 100 µL of *C. album* (2% w/w) extract solution or 1% silver sulfadiazine (SSD) (positive control). The plates were allowed to stand in aseptic environment for 2 h at room temperature for proper diffusion and then incubated face upwards at 37 °C. The zone of inhibition (mm) was measured with ruler and percent inhibition of extract with reference to drug SSD was calculated using the Eq. 1. The same procedure was repeated three time and the average values were recorded against each strain.

$$\% \text{ Inhibation} = \frac{\text{Zone of inhibition of test sample (mm)}}{\text{Zone of inhibition of standard drug (mm)}} \times 100. \tag{1}$$

Wound healing activity

Animal model

In the current study, albino $BALB^c$ mice of either sex weighing 24–32 g were used as an animal model. The animals were obtained from National Institute of Health (NIH), Islamabad, Pakistan and kept under standard conditions. The mice were kept in separate cage and fed with standard balanced diet and had a free access to water. The animals were maintained at a constant temperature (24 ± 0.5 °C) under a 12 h light/dark cycle. The animal experiment was performed after the approval by the Ethical Committee of KMU (No. DIR/KMU-EB/PA/000433), which were in accordance to the guidelines of NIH recommendations (1989). A total of 20 animals were used and divided into 4 different groups each having 5 animals. For burn wound infliction, the animals were first anaesthetized with combination of ketamine (100 mg/kg b.w.) and xylazine (10 mg/kg b.w.), followed by removal of hairs from dorsal area of mice using hair trimmer. The second degree burn was induced at shaved area by the use of specially designed round shaped pre-heated metal rod (15 mm diameter; area = 176.71 mm^2) on open flame applied perpendicularly with gravitaional force for a defined period of time i.e., 9 s. Lignocaine gel was applied on the burnt area to ensure comfort and less pain to mice (Khalid et al. 2017).

Treatment and assessment of wound healing

The ointment of *C. album* extract (2% w/w) was prepared in petroleum jelly through geometric mixing. Animals of group 1 were topically treated with petroleum jelly and considered as a vehicle control. Group 2 was topically treated with ointment of *C. album* extract, while group 3 served as a negative control group, in which mice were left untreated. Group 4 (positive control) was topically treated with reference standard drug (1% SSD). The treatment was given three times a day for 15 days. To check the efficacy of *C. album* extract, the wound area was measured and photographed on day 0, 5, 10 and 15 of the experiment and compared with vehicle, negative and positive control groups. The percent healing of various animal groups was measured using Eq. 2.

$$\% \text{ Wound healing} = 1 - \text{wound area on each day} \div \text{wound area day } 0 \times 100. \tag{2}$$

Histological analysis

On day 15, the animals were sacrificed by retro-orbitally infusing high dose of xylazine/ketamine combination. The skin tissues from wound site of all treated groups were excised and divided in two equal portions. One half were used for histological analysis and other for molecular studies. For histological analysis, tissues were fixed in 10% formalin at room temperature, unbound fixative were removed by washing in PBS. The formalin fixed tissue specimens were dehydrated by passing through gradient

alcohol of 70%, 80% and then absolute alcohol for 2, 3, and 6 h, respectively. This was followed by removal of alcohol in Xylene solution and finally, embedded in hot paraffin to prepare blocks. For slide preparation, blocks of tissue section of 5 μ thickness were cut with microtome (Robus technology, Model RM 250, 2010), mounted on slides and stained with hematoxylin and eosin (H&E) dye. The prepared slides were examined and photographed under light microscope to compare the tissue regeneration in all groups (Khalid et al. 2017).

RNA extraction and quantitative PCR

Quantitative PCR (qPCR) was performed to find out the effect of *C. album* extract on expression of healing related genes. Total RNA was isolated from skin tissues at day 15 using TRIzol (Invitrogen, US), according to the manufacturer's protocol. After purification, the RNA was used for cDNA synthesis using Revert Aid First strand cDNA Synthesis Kit (Thermo Fisher scientific, CA, USA), according to the manufacturers protocol. Then, the cDNA was subjected to qPCR for amplification of targeted (*EGF*, *PDGF*, *FGF* and *TGF-β1*) and internal control (*GAPDH*) genes, using 2X Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) in ABI Real-Time PCR System (7500 real-time PCR system) (Applied Biosystems, CA USA). The thermal cycler conditions used for qPCR included initial denaturation for 10 min at 96 °C, followed by 39 cycles of amplification each consisting of three steps: denaturation at 96 °C for 45 s, primer annealing at 55–58 °C for 45 s and amplification at 68 °C for 45 s (Table S1). The data were collected at step 3 (amplification) and analyzed for differential expression between untreated, treated (*C. album*), vehicle and positive control (SSD) groups using $2^{-\Delta\Delta Ct}$ formula.

Immunohistochemistry (IHC)

Immunohistochemistry analysis was performed to further confirm the EGFR expression. For this purpose, 4 micron tissue section was deparaffinized and immersed in a covered plastic container with target retrieval solution such as immunoDNA retriever with citrate or EDTA buffer. Then, washed with five changes of IHC wash buffer and followed by 5 min of incubation of the slides in immunoDetector peroxidase blocker. The tissue sections were covered with primary antibody according to the manufacturer instructions. The tissue sections were incubated with immunoDetector HRP label secondary antibody for 10 min at room temperature. DAB was prepared by adding one drop of ImmunoDetector DAB chromogen per mL of ImmunoDetector DAB buffer, mixed and incubated for 5 min. Finally, slides were rinsed five times with deionized water, counterstained with hematoxylin and mounted with Entellan/tissue DPX mounting media.

Statistical analysis

All quantitative pharmacological and qPCR obtained data are expressed as mean ± standard deviation. Data were analyzed using Student's *t* test for comparison between two means through sigma plot (version 2001) and *p* value was set as ≤ 0.05 for all analysis.

Results and discussion

Phytochemical analysis of *C. album* extract

It is important to know the phytochemical composition of any plant extract before conducting biological activity. The preliminary phytochemical investigation of *C. album* extract revealed the presence of major phyto-constituents like phenols, alkaloids, phytosterols, tannins, saponins, flavonoids, carbohydrates and glycosides, as summarized in Table S2. Whereas, previous studies reported the presence of all other phytochemicals except glycosides (Jain and Singhai 2012). Moreover, amino acids and proteins were not confirmed in the current examinations. It can be predicted that wound healing activity of *C. album* may be due to the indicated constituents, as study revealed that constituents such as triterpenoids, flavonoids and phenolic compounds have role in wound healing progression due to their anti-bacterial and astringent properties (Dash and Murthy 2011).

HPLC fingerprints of *C. album* extract showed major and minor peaks at retention time between 0 to 60 min (Fig. 1a and b). The resulting HPLC chromatogram at 205 nm, revealed major peaks at the retention time (min) of 3.59 and 24.62. However, several other minor peaks were also observed (Fig. 1a). In addition, at ƛ254 nm, resultant chromatogram (Fig. 1b) displayed major peaks at 3.85 (peak 1), 4.54 (peak 2), 5.63 (peak 3), 6.75 (peak 4), 10.26, 17.74 min and several other minor peaks at different retention time (Fig. 1b). It is important to note that peaks 1–4 are reported in literature for rutin, quercetin (Sanjukta and Ghosh 2012), ascorbic acid (Al-Hashemi 2014) and gallic acid (Batish et al. 2006) under similar HPLC conditions. Quercetin is an important anti-oxidant phytochemical and can promote wound contraction by enhancing fibroblast formation. Ascorbic acid enhances re-epithelialization, tensile strength and collagen expression during wound healing process (Bikker et al. 2016). Gallic acid is another anti-oxidant phytochemical that promote fibroblast and keratinocyte cell migration in both normal and hyperglucidic conditions (Yang et al. 2016). Besides, rutin, quercetin, ascorbic acid and gallic acid, there may be other important phytochemicals

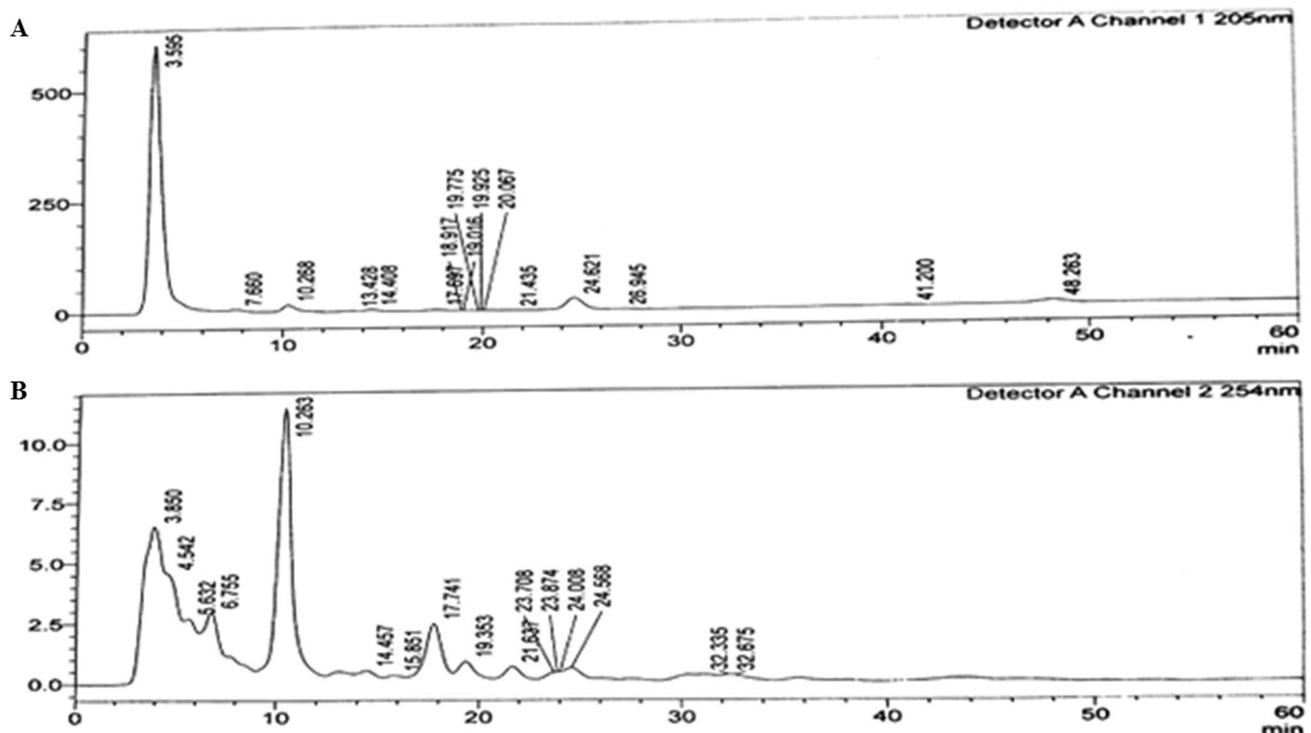

Fig. 1 The HPLC fingerprints of *C. album* extract analyzed under **a** 205 and **b** 254 wavelengths (nm). The HPLC conditions were: mobile phase (methanol: water), flow rate (1 mL/min), injection volume (20 μL), column; Shim-pack, octadecylsilane ODS (C18) analytical column (150 mm × 4.6 mm, 5 μm)

in *C. album* extract that may have role in wound healing. Therefore, *C. album* can be used to isolate bioactive compounds that promote wound healing.

Anti-bacterial assay

The anti-bacterial activity of *C. album* extract was assessed against Gram-negative and Gram-positive bacteria. The literature shows that the selected strains are predominantly associated with burn infections. The crude extract showed significant percent inhibition against Gram-negative bacterial strains *E. coli* (68.33%), *S. typhyi* (97.33%), *P. aeruginosa* (68.33%), *Klebsiella* (93.65%) and Gram-positive strains such as *B. cereus* (101.6%), *S. aureus* (88.86%), and MRSA (95.93%) in comparison to standard drug as summarized in Table 1. The anti-bacterial property of *C. album* extract is in accordance with previous research (Dwivedi and Gopal 2010). The presence of phenolic compounds in the extract attribute anti-bacterial and antioxidant properties (Mahesh Satish 2008). The preliminary phytochemical and HPLC analysis, and previous studies (Jain and Singhai 2012) indicated that this plant is rich in phenolic and flavonoid contents. Therefore, anti-bacterial activity of the *C. album* extract might be due to the presence of phenolic and/or other bioactive compounds. On this basis, *C. album* extract may help in controling burn related infections, which is one of the major reasons of mortility of burn patients.

Table 1 The zone and percent inhibition of *C. album* extract against the burn associated pathogens

Bacterial strains	Zone of inhibition in (mm)		% Inhibition
	SSD	*C. album*	
E. coli	18.0 ± 0.00	12.3 ± 0.57	68.33
B. cereus	12.3 ± 1.25	12.5 ± 1.29	101.62
S. typhyi	15 ± 4.30	14.6 ± 1.52	97.33
P. aeruginosa	18.0 ± 0.00	12.3 ± 0.57	68.33
Klebsiella	12.6 ± 0.57	11.8 ± 0.76	93.65
S. aureus	20.6 ± 4.04	18.3 ± 2.08	88.86
MRSA	12.3 ± 0.57	11.8 ± 0.76	95.93

Wound healing activity of *C. album* extract

After injury, wound healing is an immediate physiological response, which replaces damaged cellular structures with healthy new skin tissues (Ghayempour et al. 2016). To assess the effect of *C. album* tratment on the healing process, wound area reduction was measured and photographed on

day 0, 5, 10 and 15. A sequential pattern of healing was observed in animals treated with *C. album* extract and positive control. The wound area was reduced from 176.71 ± 0 mm^2 (day 0) to 71.8 ± 7.40 mm^2 (day 15) in *C. album* extract treated group and from 176.71 ± 0 mm^2 (day 0) to 32.59 ± 1.30 mm^2 (day 15) in positive control group. The results have been summarized in Table 2. In comparison, the vehicle treated group showed slight increase in wound area from 176.71 ± 0 mm^2 (day 0) to 231.62 ± 13.42 mm^2 (day 10) of post wounding after which, shrinkage in wound size started and reduced to 157.67 ± 4.46 mm^2 on day 15 of the experiment. Similarly, negative group also showed slight increase in wound area from 176.71 ± 0 mm^2 (day 0) to 197.3 ± 17.3 mm^2 (day 10) after which, wound size was partially reduced to 137.17 ± 18.96 mm^2 on day 15 as shown in Fig. 2 and Table 2. Thus, percent healing of vehicle, *C. album* and positive control were 11%, 60% and 82%, respectively.

Plant extract can enhance the rate of wound healing through multiple mechanisms such as by enhancing extracellular matrix proteins, growth factors, cell division, maturation and migration of many cells involved in healing (Shukla et al. 1999). The *C. album* extract may exert wound healing effects through the above mentioned mechanisms. For example, *C. album* has high level of glycosaminoglycans (Martini and Brzezinski 2018), which can enhance the collagen synthesis, which play key role in tissue regeneration (Martini and Brzezinski 2018).

Table 2 The average wound area contraction during the course of treatment in *C. album* extract, vehicle, negative and positive control groups

Group name	Average burn wound area in mm^2			
	Day 0	Day 5	Day 10	Day 15
Vehicle control	176.71 ± 0	303.95 ± 17.68	231.62 ± 13.42	157.67 ± 4.46
C. album	176.71 ± 0	273.05 ± 10.87	289.87 ± 4.72	71.8 ± 7.40***
Negative control	176.71 ± 0	201.87.3 ± 22.91	197.3 ± 17.3	137.17 ± 18.96
Positive control	176.71 ± 0	197.19 ± 6.85	123.24 ± 1.64	32.59 ± 1.30

$p \leq 0.05$ was considered statistically significant (*represents significant statistical differences $p \leq 0.001$***, $p \leq 0.01$**, $p \leq 0.05$*)

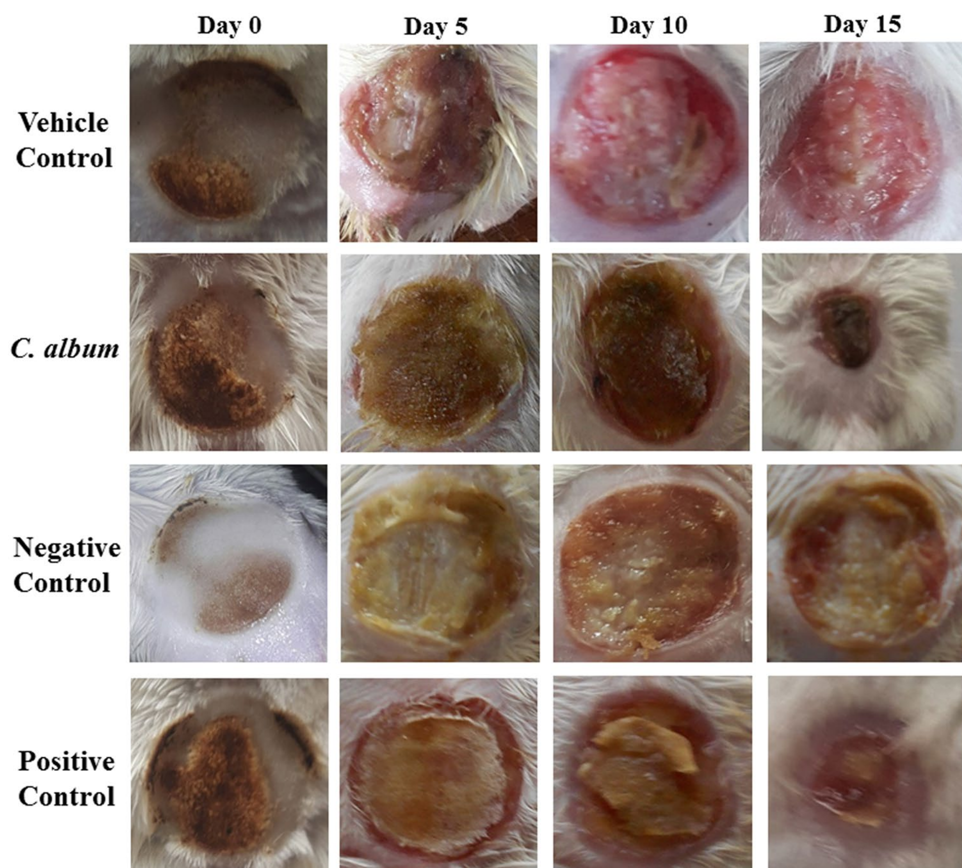

Fig. 2 Representative burn wound photographs of animals treated with 2% (w/w) *C. album* extract, vehicle, negative and positive control groups on day 0, 5, 10 and 15. It can be clearly seen in the photographs that *C. album* extract enhanced the wound healing as compared to vehicle

Therefore, it may be proposed that *C. album* based products may find potential in the treatment of burn wounds.

Histopathological studies of skin tissue

Histological analysis is one of the most definitive and consistent approach to check the progress of tissue regeneration (Sajjad et al. 2019). The normal anatomy of skin tissues stained with H&E showed healthy granulation, hair follicles, epithelial cells, fibroblast cells, blood vessels, epithelial, epidermal and dermal layers (data not shown). On the other hand, histological analysis of *C. album* extract treated mice skin showed tissue regeneration, granulation, neovascularization and re-epithelialization with no ulceration slough. Moreover, healthy hair follicles and sebaceous glands are also observed (Fig. 3), which clearly indicated the effective healing pattern in extract treated group. The histological examination of vehicle and un-treated groups showed ulceration slough, necrotic tissue and incomplete re-epithelialization. Moreover, bacterial colonies, inflammatory cells like lymphocytes, macrophages and plasma cells were observed in high power resolution images of vehicle and un-treated control group, which clearly indicates the aberrant inflammatory stage (Fig. 3). On the other hand, animals treated with reference standard drug showed regenerated epithelium with skin appendages, indicating good healing. Phytochemicals like phytosterols can stimulate tissue regeneration by attracting macrophages and enhancing collagen deposition (Harish et al. 2008). Therefore, it is suggested that *C. album* might enhance the tissue regeneration through-above mentioned mechanism.

Quantitative PCR analysis

As the histological examination of *C. album* extract treated tissues illustrated remarkable increase in epidermal and dermal regeneration, endothelial cell proliferation, neovascularization and rapid wound area contraction as compared to the control groups. For further confirmation, qPCR analysis were performed to check the expression level of healing related genes, *EGF, FGF, TGF-β1* and *PDGF*.

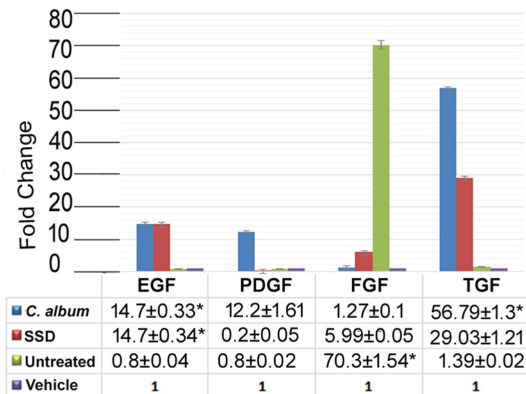

Fig. 4 Comparative expression analysis of *EGF, PDGF*, *EGF*, *TGF-β1* in *C. album* treated, positive control, negative control and vehicle groups on the day 15 of the experiment. Fold change in expression of genes is shown on Y axis and names of the genes on X-axis. Table below explains the values of fold change in gene expression with standard deviation. $p \leq 0.05$ was considered statistically significant (*represents significant statistical differences < 0.001)

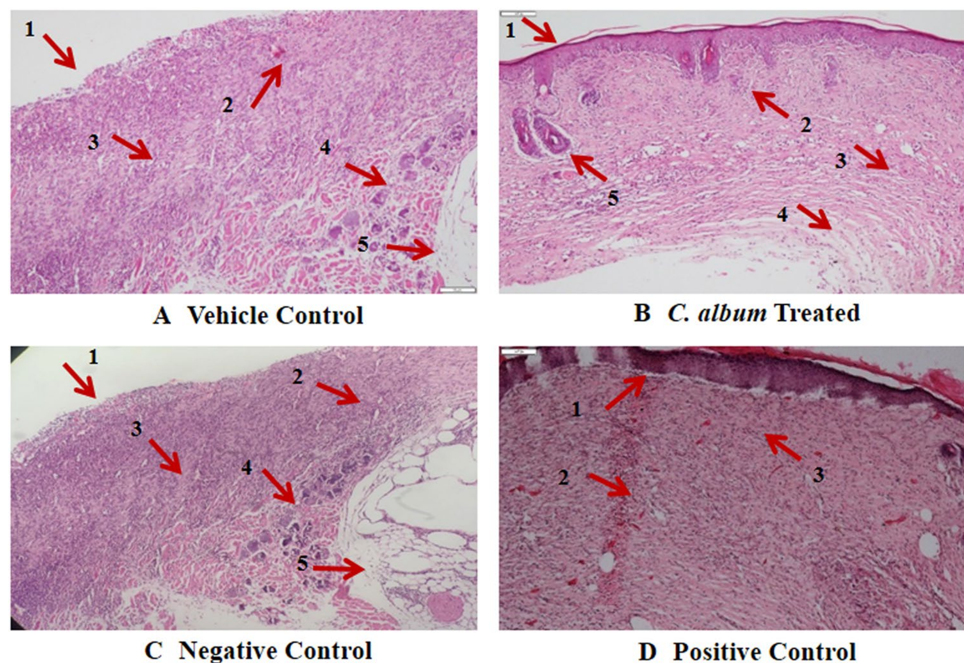

Fig. 3 Histological evaluation of skin tissues stained with hematoxylin and eosin (H&E) on day 15. In the **a** vehicle group, arrows showed as (1) No epidermis (2) Inflammation (3) Granulation tissue (4) Bacterial colonies and (5) Adipose tissue. In **b** burn group, arrows showed as (1) Epidermis (2) Skin adnexa (3) Fibrosis (4) Muscle (5) Hair follicle. In **c** negative burn group exhibit the same results as vehicle group, while **d** positive control burn group also revealed (1) epidermis (2) fibrosis (3) Skin adnexa

Results showed increase in expression level of *EGF, FGF*, *TGF-β1* and *PDGF* genes in *C. album* extract treated as compared to vehicle control group (Fig. 4). The highest expression in treated group was observed for *TGF-β1* gene, where the treated group showed 56 times higher expression as compared to vehicle, and almost double to SSD group, while the untreated expression (1.4) was closed to vehicle group. The effect of *C. album* treatment was almost similar to SSD for *EGF* gene, where the fold change in expression was 14.7 and 14.8, respectively; the untreated group (0.79) expression was closer to vehicle expression level (1). In case of *PDGF* expression, a significant increase in treated group (11.6) was observed as compare to vehicle, however, the SSD effect on PDGF was very low (0.11); while the untreated expression (0.85) was closed to the vehicle group expression. Unexpectedly, there was almost no effect of *C. album* (1.28) on *FGF* expression, however, the SSD effect was significant (21); the untreated group shows highest expression (73) as compare to vehicle group. Therefore, current experimental results suggested that the topical administration of *C. album* extract thrice a day may activate the genes for effective healing.

Immediately after injury, platelet degranulation leads to the release of PDGF, which stimulate mitogenicity and chemotaxis of neutrophils, macrophages and fibroblasts cells to the injured area (Velnar et al. 2009). Furthermore, it also stimulates macrophages to synthesize and release FGF growth factor (Velnar et al. 2009). Several studies report proliferative role of EGFR, as it speeds up the keratinocyte proliferation and cell migration to the wound sites. While, FGF promote fibroblast and keratinocyte motility along with granulation tissue formation, tissue regeneration and re-epithelialzation. Moreover, experimental findings suggest that *TGF-β1* may define transition from inflammatory to immunoregulatory phase during the healing process (White and Mitchell 2000). In addition, it affects the maturation and organization of the extracellular matrix (ECM) in the wound (King 2014). Likewise, both *PDGF* and *TGF-β1* can also improve wound area closure by converting fibroblast into myofibroblast (Barrientos et al. 2008). Studies suggested that antioxidants upregulate the expression of several growth factors including *FGF, TGF-β1, PDGF* and *EGF* (Kalay and Cevher 2012; Avsar et al. 2016; Zduńska et al. 2018). The product of these genes work in coordination during the course of healing. It is suggested that antioxidants present in *C. album* extract may have upregulated these genes which ultimately improved wound healing (Fig. 5).

Our experimental results corroborated that *C. album* extract significantly up regulated the expression of *TGF-β1, PDGF* and *EGF* as compared to vehicle control group. However, there was no significant effect of *C. album* on expression level of FGF. The histological observation showed thicker keratin and epidermal layer in *C. album* treated groups as compared to the control groups, which may be due to up-regulation of *TGF-β1* and *PDGF*.

Immunohistochemistry

Epidermal growth factor receptor (EGFR) are considered to have essential role in skin integrity and wound healing. Several studies reported that EGF topical treatment improved wound healing that may activate EGFR signaling (Bodnar 2013). It is also believed that EGFR play a role by enhancing migration and proliferation that is essential for normal wound healing. This discussion depicts the importance of EGFR signaling in proper healing of wounds. The anti-EGFR antibody immunohistochemical staining of *C. album* extract treated tissues displayed more intense staining compared to the vehicle control group (Fig. 6). Therefore, the effect of topical administration of crude extract on wound healing has potential to accelerate the tissue regeneration and re-epithelialization due to up regulation EGFR expression. These observations are is accordance with previously reported study (Leydon 2014) in which the activation of EGFR was observed in acute phase of wound healing.

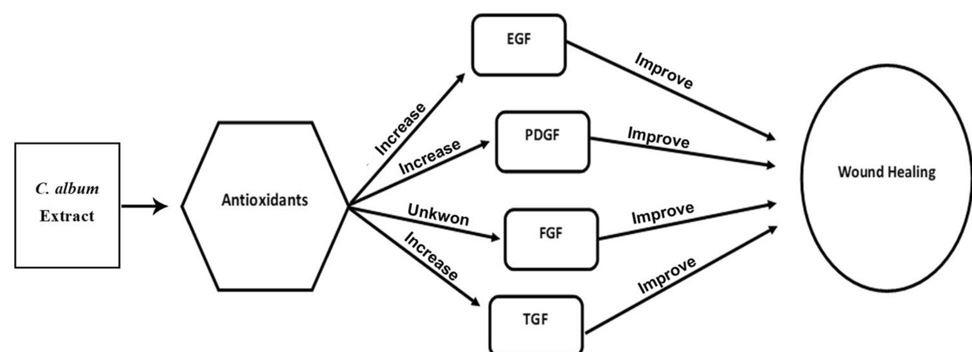

Fig. 5 Schematic representation of the possible mechanism of the *C. album* extract on wound healing. *C. album* extract have antioxidants, that may upregulated the studied genes (*EGF, PDGF*, *EGF*, *TGF-β1*) that ultimately improved the wound healing

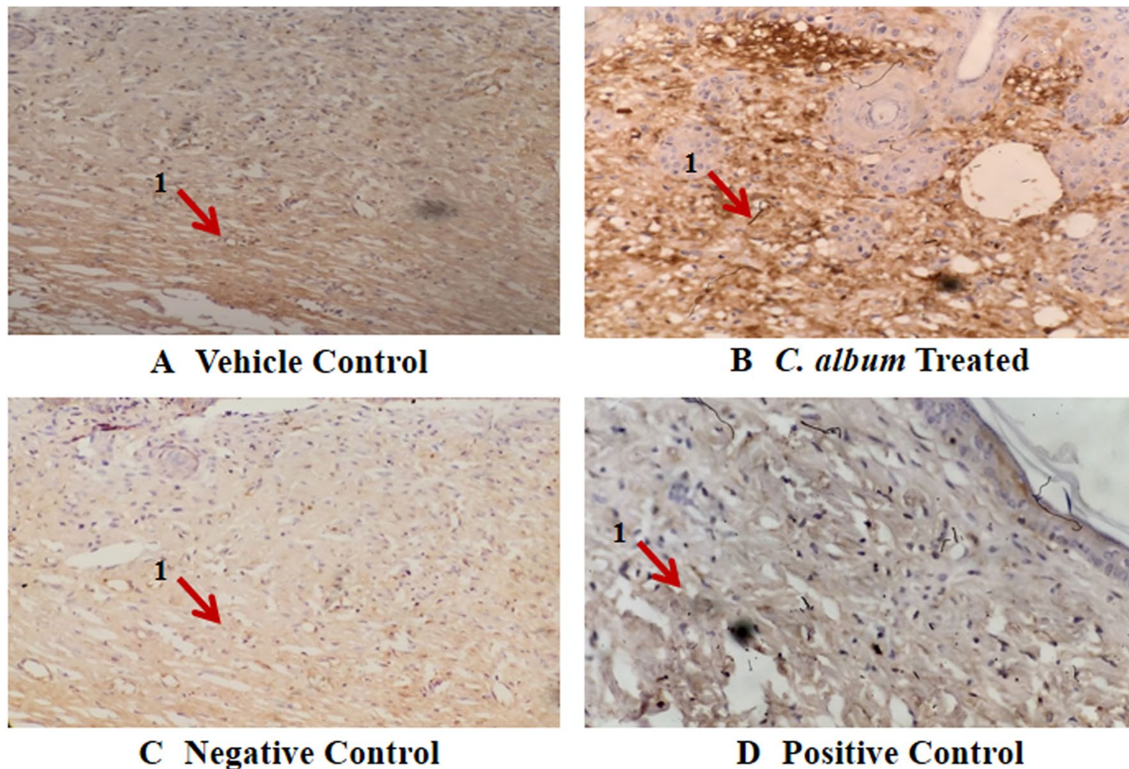

Fig. 6 Representative immunohistochemical microphotographs of mice tissues (400 ×) of **a** vehicle group, **b** *C. album* extract group, **c** negative control group, **d** positive control group. EGFR was not expressed in the vehicle and negative control groups. In the extract treated group, the EGFR showed strong expression and in positive group have weak expression

Conclusion

The current study concluded that *C. album* extract is a rich source of all major phytochemical groups including phenols, alkaloids, phytosterols, tannins, saponins, flavonoids, carbohydrates and glycosides. The HPLC chromatogram also showed the presence of various comparable peaks of bioactive compounds that may have role in wound healing and tissue regeneration. Moreover, *C. album* extract showed anti-bacterial activity against burn associated pathogens such as *E. coli*, S. *typhyi*, *Klebsiella*, *P. aeruginosa, B. cereus*, *S. aureus*, and MRSA. The treatment of *C. album* extract enhanced the wound healing via up regulation of wound related genes. The results of histological analysis revealed better tissue regeneration in extract treated animals. Therefore, further studies should focus on isolating the wound healing-related bioactive compounds from the *C. album* extract. There is a need to conduct further studies to understand the complete molecular mechanisms of wound healing and tissue regeneration effects *C. album* extract.

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Author contributions SS, TK and FW conceived the project, supervised the research, and review the manuscript. AS, NN, AJ and HMR designed and performed the experiments, analyzed the results, wrote the manuscript as well as prepared the Figures. WS assisted in wound healing and histopathology studies, and manuscript writing. KS assisted and supervised the molecular studies and results analysis. KS also help in writing of the manuscript. All authors read and proofread the manuscript.

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Data availability Full data is available on reasonable request.

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

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