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Wound healing potential of licorice extract in rat model: Antioxidants, histopathological, immunohistochemical and gene expression evidences

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ABSTRACT

Wound healing is a public health concern. Licorice gained a great attention for its antioxidant and anti-inflammatory properties which expand its valuable effects as a herbal medicine. In this study, we pointed out to the wound healing potential and the mechanism by which licorice alcoholic extract can modulate cutaneous wound healing through immune, antioxidant, histopathological, immunohistochemical (IHC) and molecular studies. 24 Wister rats were assigned into 3 groups (n = 8 each); control group, topical and oral supplied groups. Licorice extract administration significantly increased total and differential leucocyte counts, phagocytic activity of neutrophils, antioxidant biomarkers as superoxide dismutase (SOD), glutathione peroxidase activities (GPx) and reduced glutathione (GSH) content with a notable reduction in oxidative stress marker malondialdehyde (MDA). Moreover, histopathological findings detected complete re-epithelialization with increasing collagen synthesis while IHC results revealed a significant enhancement in the expression of α-SMA, PDGFR-α, FGFR1 and Cytokeratin 14 in licorice treated groups compared with the control group. Licorice extract supplementation accelerated wound healing by increasing angiogenesis and collagen deposition through up-regulation of bFGF, VEGF and TGF-β gene expression levels compared with the control group. UPLC-PDA-MS/MS aided to authenticate the studied *Glycyrrihza* species and recognized 101 potential constituents that may be responsible for licorice-exhibited potentials. Based on our observations we concluded that licorice enhanced cutaneous wound healing via its free radical-scavenging potential, potent antioxidant activities, and anti-inflammatory actions. Therefore, licorice could be used as a potential alternative therapy for wound injury which could overcome the associated limitations of modern therapeutic products.

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1. Introduction

The skin represents a primary defense against external pathogen invasion and environmental extremes [1]. The immune system acts as a master key that orchestrates wound healing process via the collaborative efforts of numerous pro-angiogenic cells including fibroblasts, leukocytes, endothelial cells, and epidermal cells [2–4]. Immediately after the skin injury, hemostasis stage (blood clotting) is initiated and platelets aggregation is enhanced which subsequently produce various chemotactic factors as TGF-ß1, TGF-ß2 and PDGF. Then, inflammatory cells (neutrophils, lymphocytes, and macrophages) are recruited to protect the wound from infection [5]. Keratinocytes, macrophages, platelets, and endothelial cells of wounded area release some growth factors such as EGF, FGF, PDGF, VEGF, TGF-β; cytokines (IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ) and chemokines, which control other subsequent stages of wound healing [4,6]. Wound repairing occurs and progresses for varying lengths of time based on the extent of the injury. However, healing may be severely complicated in a way that perturbs patient's life quality. Therefore, several studies have been conducted to mitigate these complications and to find new inexpensive and innovative treatments to accelerate wound closure with fewer breakdowns [7,8]. There is an urgent need to discover an extra-body assistance to speed up the healing process and restore skin integrity while lowering the associated costs [9,10].

Natural herbs represent safe natural source of several bioactive constituents with minimal side effects as new treatment suggestions for cutaneous wound healing [11]. Herbal medicine is less expensive compared with synthetic drugs. Some researchers focused on the healing potential of medicinal herbs and worked hard to understand these herbs in depth through mechanistic studies [12,13]. There are various reports on herbal application for skin injuries healing [14,15].

Licorice (Family: *Papilionaceae/Fabaceae*) is a traditional medicinal sweet and soothing herb which remains one of the most widely used herbs as a natural sweetener, an additive for flavoring and sweetening candies and beverages in many countries [16] and also in skin-cosmetics [17].

Licorice root has several bioactive components, most of which fall into the chemical classes of triterpenoids and flavonoids [18]. Wang et al. [19] documented that licorice is considered a source of amino acids, proteins, simple sugars, polysaccharides and mineral salts, pectins, resins, starches, sterols, and gums. In addition to its content of tannins, phytosterols (sitosterol and stigmasterol), coumarins, vitamins (B1, B2, B3, B5, E, and C), and glycosides. Besides pantothenic acid, lecithin, biotin, niacin, manganese, calcium, calcium salts, proteins, and nucleic acids which may aid in wound healing [20]. Furthermore, Rizzato et al. [21] and Wang et al. [19] isolated numerous biological compounds from licorice such as triterpene and saponins which are responsible for sweet taste of licorice that is almost 50 times sweeter than sucrose and are being the primary active ingredient [22]. Flavonoids content is responsible for licorice yellow color although only little flavonoids are well identified [23]. The flavonoids are basically considered as antioxidant and anti-inflammatory agents. Licorice extracts have the ability to down-regulate expression of pro-inflammatory cytokines as tumor necrotic factor alpha (TNFα), interleukin 1 (IL-1) and interleukin 6 (IL-6) [22]. In traditional medicine, the roots and rhizomes of licorice were efficiently used clinically for centuries for their antifungal [24]; antimicrobial efficacy [25], antiviral and antitumoral activity [26]; anti-bacterial [27]; antioxidant [28]; antiulcer [29], improve wound healing of gastric and oral wounds [30,31]; as well as healing of colitis mucosal ulcers [29].

Commercially available licorice may be a mixture of two or more species. *Glycyrrihza* genus comprises several species (about 30). Commercial sources of licorice are Iraq, Turkey, Iran, Russia, Spain and China. The most identified and well-studied species are *G. glabra*, originating from the Mediterranean region, *G. uralensis* and *G. inflata* from China [32]. It is not easy to differentiate between different species based on the morphology of roots. Genetic identification may lead to misidentification as pointed out by Liao et al. [33]. *Glycyrrihza* species share many chemical constituents. However, chemical profiling studies identified the key compounds that are species determinants [21,33–36].

Based on the aforementioned facts about the licorice properties, this study was designed to evaluate the positive effects of licorice as an alternative therapy for wound healing, besides exploring its mechanism for wound healing potential in rat wound model. Moreover, UPLC-PDA-MS/MS analysis was used in this study for chemo-taxonomical characterization and authentication of the plant species.

2. Materials and methods

2.1. Ethical approval

The experiments were approved by the Institutional Animal Care and Animal Ethics Committee, Faculty of Veterinary medicine, Kafrelsheikh University, Egypt. All precautions were followed to diminish animal suffering during the experiment.

2.2. Plant materials and extract preparation

One kilogram of Licorice roots was collected from Haraz area, Abdeen, Cairo, Egypt. The plant was kept in the Herbarium in the Department of Pharmacognosy. The plant roots were ground to obtain powder and then soaked in 70% ethanol for four days then filtrated and the filtrate was concentrated under vacuum to remove the alcohol, the aqueous residue was kept in the refrigerator during the experiment, and administered to each rat at a dose of 4 g/kg BW in powdered form.

2.3. UPLC-PDA-MS/MS for metabolite analysis

Powder of commercially available Glycyrrihza (100 g) was extracted with 70% aqueous ethanol. Then, the filtered extract was evaporated under vacuum to yield a brown aqueous extract which was further lyophilized to obtain a brown residue used for metabolite analysis. UPLC was carried out using a Nexera-i LC-2040 liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with UPLC shimpack velox C18 Column, 2.1 × 50 mm; 2.7 μm particle, using the following gradient (solvent A: water containing 0.1% formic acid; solvent B: acetonitrile) at flow rate of 0.2 mL/min: 0–2 min: 10% B; 2–5: linear gradient to 30% B; 5–15 min: linear gradient to 70% B; 15–22 min: linear gradient to 90% B; 22–25 min: linear gradient to 95% B; 25–26 min: linear gradient to 100% B; 26–29 min: isocratic 100% B; 29–30 min: linear gradient to 10% B. The sample (2 mg/mL) was prepared by dissolving the extract in HPLC methanol followed by filtration through 0.2 μm membrane disc filter, and the resultant solution was injected (3 μL) into the system. Detection was accomplished by using a LC-2030/2040 PDA detector and a LC-MS 8045 triple quadruple mass spectrometer equipped with an electrospray ionization (ESI) source in negative and positive mode (Shimadzu, Kyoto, Japan) applying the following settings: nebulizer gas N_2, 3 L/min, 4 bar; dry gas N_2, 10 L/min, 400 °C; capillary voltage − 4 kV; endplate offset − 4.5 kV; collision energy 8 eV (Full MS) or 20–35 eV (MS/MS).

2.4. Determination of the total content of flavonoids and polyphenols

Serial dilutions of the extract were colorimetrically assayed using the aluminium chloride method and rutin as a standard for determining the total flavonoid content [37]. The total content of polyphenols was measured following Folin-Ciocalteu method and gallic acid as a standard [38]. The measured contents were expressed as mg/g equivalent of the corresponding standard for each method.

2.5. Experimental animals

Twenty four male Wister rats (10 weeks age; weighing 200 ± 20 g) were obtained from the Animal House Colony of the Tanta Center. The animals were divided into three equal groups (8 rats per group). The animals were fed a standard diet (Al Wadi Co., Giza, Egypt) and provided water ad *libitum*. To prevent biting and possible wound scratching from each other; each rat was housed in separate plastic cage. All animals were allowed to acclimatize to laboratory conditions [temperature 22–25 °C, relative humidity 50–60%, and 12-h photoperiods (lights on 07:00–19:00 h)].

2.6. Experimental design

After one week as an adaptation period, all rats (n = 24) were randomly assigned into 3 groups (8 rats per group); control group without treatment, topically-applied group on which licorice alcoholic extract was topically applied on the back of examined rats and orally-given group with licorice alcoholic extract by stomach tube (Fig. 1A).

2.7. Establishment of full thickness dermal excisional wound

The rats were anesthetized with an intra-peritoneal injection of ketamine-xylazine combination (ketamine 70 mg/kg and xylazine 7 mg/kg) for anesthetizing the rats. Then, the back hair was shaved and disinfected with 70% ethanol. Full-thickness skin wound excision measuring 1.5 × 1.5 cm was made on the back of each animal according to Atiba et al. [39]. Wound area size was measured and photographed with special size analysis software, NIH Image J software downloaded from http://www.rsb.info.nih.gov/ij) on the days 0, 3, 6, 9, 12, 15 and 18 post-wounding (PW). The change in wound size was expressed as a percentage to the original wound size (day 0) and shown in Fig. 1. The medical dressing was changed to all groups in the above-mentioned days of the experiment with inspection of the wound healing degree. The control groups did not receive any treatment along the course of the experiment only Band-Aid to keep the wound moist and avoid bias in the results.

Experimental scheme, schedule time of wounding, wound area measurement, licorice alcoholic extract administration and sampling were portrayed in Fig. 1.

2.8. Leukogram, immunological and antioxidant parameters

At the 18th day post wounding (DPW) blood samples were collected via retro-orbital bleeding under light ether anesthesia (Sigma Chem. Co., St Louis, Mo. U.S.A). Two blood samples were collected from each rat. One half was transferred to an eppendorf tube containing the anti-coagulant heparin (20 IU/mL) for determination of the total and differential leukocyte counts by using automated blood cells counter with an Auto Hematology Analyzer (Sysmex F-800, Japan) according to Buttarello [40], phagocytic activity and phagocytic index of neutrophils using *Candida albicans* by following Kawahara et al. [41]. The other half of the blood sample was collected in an eppendorf tube without anti-coagulant, the blood samples were then allowed to coagulate before centrifuging at 3000 rpm for 15 min at 4 °C, then were subjected to biochemical determination of the malondialdehyde (MDA) content according to Ohkawa et al. [42] and the superoxide dismutase (SOD) activity using the techniques outlined by Nishikimi et al. [43]. The glutathione peroxidase activity (GPx) was evaluated according to Paglia and Valentine [44] and reduced glutathione (GSH) content using the

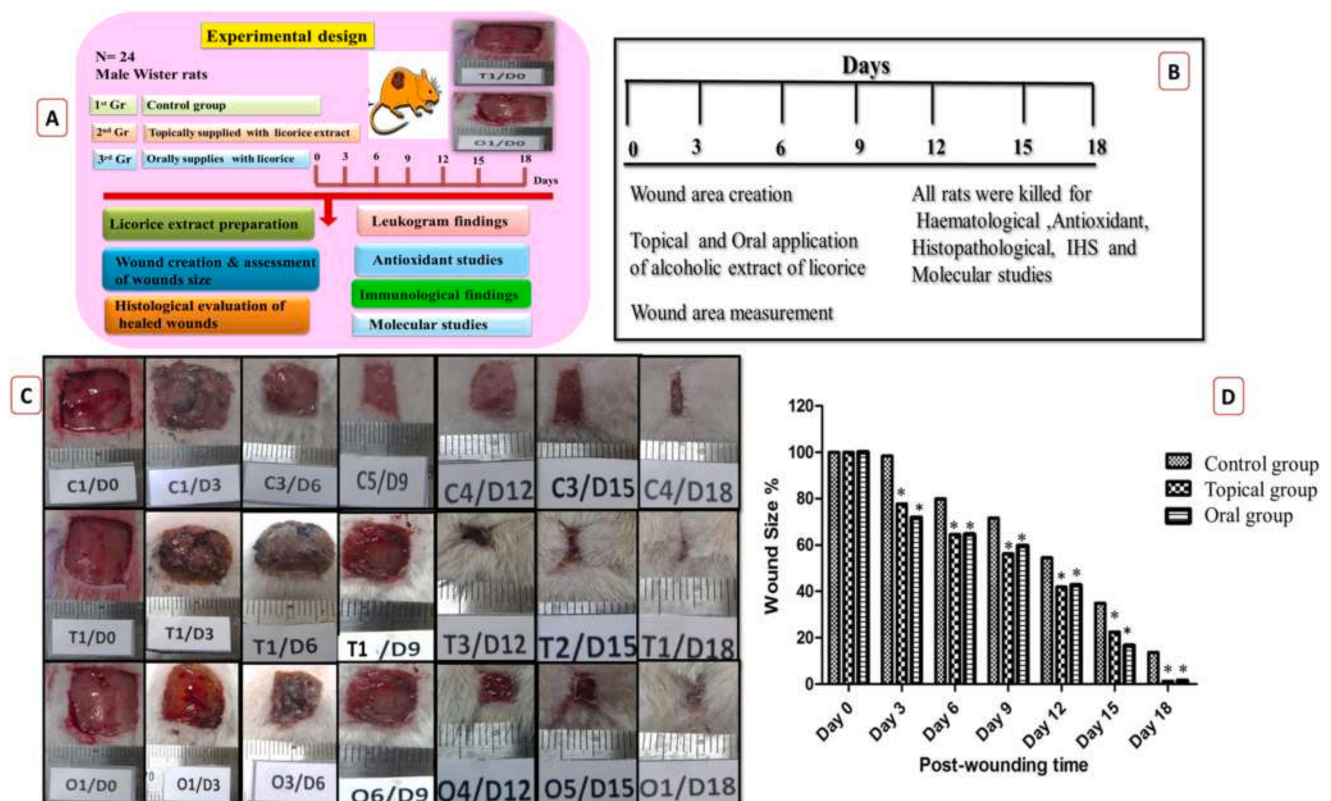

Fig. 1. (A) Experimental design (B) Schedule time of wound area measurement, liquorice oral and topical application and sampling (C) Photographs of the gross appearance of full-thickness skin wounds at various time points after treatment without or with topical and oral Liquorice alcoholic extract application on wound area. (D) The percentage (%) of wounds area on days 0, 3, 6, 9, 12, 15 and 18 post wounding as compared with wound closure in control group. The wound closure rate was expressed as the percentile of wound area compared with that on post wounding day 0 (100%).Values are mean ± SE ($P < 0.05$) [C = control group, T = topical group, O = oral group].

techniques outlined by Owens and Belcher [45], all tests were obtained from Biodiagnostic Co. (MyBioSource Inc., San Diego, California, USA) and were performed following the manufacturer's instructions.

2.9. Collection of tissue specimens

After the end of the experiment, 18 DPW, all rats were sacrificed by decapitation under anesthesia with an intra-peritoneal injection of pentobarbital anesthesia (500 mg/kg). The entire wound including a margin of approximately 5 mm of normally un-wounded skin areas were excised. These samples were divided into 2 halves; one sample collected in 10% neutral buffered formalin for histopathological and immune-histochemical (IHC) examinations. While the other half was collected in 2 mL sterile eppendorf tubes and shocked in liquid nitrogen before storage at − 80 °C until being used for RNA extraction.

N. B: After the end of the experiment, all the scarified rats together with remnants of tissue samples and bedding materials were buried in strictly hygienic and properly controlled constructed burial pit.

2.10. Histological evaluation of healed wounds

Specimens from the full thickness wound tissues, including the adjacent normal skin, were taken from 5 rats from each group at 18 DPW and were immediately fixed in 10% neutral buffered formalin for 24 h, routinely processed and embedded in paraffin blocks [46]. The 4 μm-thick sections were stained with haematoxylin and eosin (H&E) and examined microscopically. H&E-stained sections were examined for the extent of epidermal re-epithelisation, inflammatory cells including polymorphonuclear leukocytes (PMNL), macrophages and lymphocytes, fibroplasia, and newly formed vessels (Angiogenesis).

2.11. Immunohistochemical analysis & scoring of immunoreactivity

Immunohistochemical staining (IHC) was performed on all skin tissues examined histologically using 4-μm thick paraffin-embedded sections. All tissue samples were stained at the same time with the same procedure. In brief, after de-waxing in xylene and rehydration in graded ethanol, the sections were then treated with a solution of hydrogen peroxide 0.3% in methanol for 20 min at room temperature (RT) to inhibit endogenous peroxidase activity. For antigens retrieval, the sections were immersed in Target Retrieval Solution High pH (Dako®) and autoclaved at 121 °C for 15 min. To prevent the binding of non-specific proteins, the sections were incubated with Protein Block Serum Free (Dako®) for 30 min at RT. Sections were then incubated with the following primary antibodies: monoclonal mouse Anti-α-SMA (Dako, Dako-Cytomation, Carpinteria, CA) at 1:20 dilution; rabbit polyclonal antibody against PDGFR-α (SC-338, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:100 dilution; goat anti-human polyclonal Anti-FGFR1 (LifeSpan BioScience) at 1:500 dilution and monoclonal mouse Anti-K14 (Thermoscientific, CA, USA) at 1:50 dilution. Incubations were carried out overnight. Following the application of the secondary antibody: monoclonal anti-mouse IgG; anti-rabbit IgG and monoclonal anti-goat IgG (EnVision + System HRP; Dako) for 30 min at room temperature, the bound peroxidase was detected with liquid DAB substrate chromogen system (DAKO). The sections were then washed in distilled water, counterstained with Mayer's haematoxylin, dehydrated in an alcohol gradient, cleared with xylene, and mounted for examination under light. The positive findings of samples were demonstrated with brown color under a light microscope. For each case, negative control slides consisted of sections incubated with antibody vehicle or no immune rabbit or mouse serum [47].

Tissue specimens from ten sections were analyzed by immunohistochemistry for the presence of a set of markers associated with myofibroblasts phenotypic differentiation and epidermal activation and proliferation. Several target proteins turned out to be suitable to monitor wound healing including α-SMA positive myofibroblasts, FGFR1, PDGFR-α and cytokeratin 14.

α-SMA, PDGFR-α and FGFR1 immunolabeling were quantitatively analyzed in blind mode by a pathologist using optical microscope (40× Leica Microsystems AG, Wetzlar, Germany). Five animals were evaluated in each group. A total of ten fields per section; two from the right margin of the wound, two from the left margin of the wound and six from the center (wound bed) of the wounds, in 2 slides per animal, were examined and analyzed at a magnification of × 400 (× 40, 10 ocular). For quantitative analysis, the overall number of evaluated cells per section was 900–1000 cells. The cytokeratin14-stained samples were visualized under microscope at different magnifications. From the obtained image, epidermal thickness was measured with Image J analysis software (National Institutes of Health, MD, USA) by considering the thickness at ten different locations along the re-epithelialized skin wounds on day 18.

2.12. Total RNA extraction and reverse-transcription polymerase chain reaction

Collected tissue samples from the wound areas shocked in liquid nitrogen then stored at − 80 °C were used for RNA extraction using TriZol reagent (iNtRON Biotechnology). The quality and concentration of the extracted RNA were assessed using a Nanodrop ® BioDrop spectrophotometer. The A260/A280 ratio was used to determine the quantity of total RNA. The ratio of A260/ A280 was from 1.8 to 2.0. The integrity of all extracted RNA was checked by electrophoresis in 1.5% ethidium bromide-stained denaturing gel (Sigma, Germany) at 100 V in 1x Tris-acetate acid-EDTA (TAE) buffer, pH 8.0. The gel image was visualized using UV transilluminator (azure c200).

2.13. cDNA synthesis and quantitative real-time PCR of b-FGF, VEGF, TGF-β1

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression of rat-specific primers for *bFGF*, *VEGF*, *TGF-β1* and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the house-keeping gene were performed using primers in Table1 [48–50]. 2 μg of total RNA was reverse transcribed to first-strand cDNA using the Intron-Power cDNA synthesis kit (Cat. No. 25011) according to the manufacturer's instructions. The cDNAs were used as the template for RT-PCR using SYBR in the Mx3005P Real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The relative differences in gene expression were calculated using threshold cycle (CT) values that were first normalized to those of the Albino Rat (*Rattus norvegicus*) GAPDH house-keeping gene and using ΔCT value of control samples as calibrator using the $2^{-\Delta\Delta CT}$ method as previously described by Livak and Schmittgen [51]. All samples were performed and analyzed in triplicate.

2.14. Statistical analysis

The statistical significance between the different tested groups was evaluated by multiple *t*-tests (unpaired two-tailed *t*-test) with Holm-Sidak method for correction of multiple comparisons using GraphPad Prism software version 8.00 (GraphPad Software, San Diego, California USA). Unpaired Welch *t*-test was used for relative gene expressions of b-FGF, VEGF, TGF-β1 and α-SMA data analysis. P value < 0.05 was considered statistically significant. All data were tabulated as means ± SD.

Both H&E and immunohistochemistry analysis results were compared between groups by one-way analysis of variance ANOVA test with post hoc Tukey. The level of significance for significant difference between groups was set at $P < 0.05$ in all analyses.

3. Results

No animals' mortalities were recorded during the whole period of the

Table 1
Primer sequence of selected genes used in RT-PCR analysis.

Gene	Primer sequence 5′–3′	Gene bank accession number	Annealing temperature	Refs.
GAPDH	5′-CAGCAATGCATCCTGCAC-3′ 5′-GAGTTGCTGTTGAAGTCACAGG-3′	XM_017592435.1	60 °C	[48]
VEGF	5′-AGGCTGCACCCACGACAGAA-3′ 5′-CTTTGGTCTGCATTCACATC-3′	NM_001110333.2	55 °C	[49]
bFGF	5′-CGATAGAACACGGCATCAaTC-3′ 5′-CATCAGGCAGTTCGTAGCTC-3′	NM_019305.2	50 °C	[50]
TGF1β	5′-CCAGATCCTGTCCAAACTAA-3′ 5′-TTTTGTCATAGATTGCGTTG-3′	X52498.1	53 °C	[50]

Vascular endothelial growth factor: (VEGF); Basic fibroblast growth factor:(bFGF); transforming growth factors beta: (TGF-β).

study.

3.1. Characterization of the detected compounds and plant species

The analysis of the data in the negative and positive ion modes (Fig. 2) revealed the presence of 101 compounds. The complete chemical fingerprint is enlisted in Table 2. UPLC-MS data were processed using Shimadzu's Lab Solutions software. The compounds were identified based on the molecular weight determined from the most intense adduct ion found in the full MS spectrum of each compound, MS/MS fragmentation ions, or neutral losses found in full MS or MS/MS spectra and, whenever possible, maximum absorption wavelength from PDA spectra. MassBank and FooDB databases were used as references in identifying the eluted compounds in addition to the published data in the literature. Detailed description of all bioactive compounds listed in Supplementary file 1.

Moreover, some dimer compounds were identified in *Glycyrrhiza* species. In this work dimers of glabrone + glabridin, glabridin + sophoracoumestan A and flavonoid dimers were detected at [M-H]⁻ ions at *m/z* 659, 657 and 673, respectively and their fragmentation profiles matched the published data [21].

Species chemical makers were proposed by Scalabrin [32], Farag et al. [34] and Rizzato et al. [21] They identified glabridin, glabrol, 3-hydroxyglabrol, kanzonol Y as markers for *G. glabra*; glycycoumarin for *G. uralensis* and licochalcone A and glyinflanin A for *G. inflata*. Our results indicated the detection of these markers in the extract of the licorice sample used in this study confirming that three species namely *G. glabra*, *G. uralensis* and *G. inflata* mediated the observed biological effects in this work.

3.2. Total content of flavonoids and polyphenols

The major identified content of total flavonoids was determined as 47.00 mg/g equivalent to rutin while the content of total polyphenols was measured as 70.34 mg/g equivalent to gallic acid. Indicating that the 70% alcohol extract is rich in polyphenols and flavonoids.

3.3. Licorice alcoholic extract in relation to wound healing

Skin wound healing was determined by the percentage of wound surface covered by regenerated epidermis in rats of control, topical and oral groups as presented in Fig. 1C. Which demonstrates that, the wounds treated by licorice extract recovered much faster with better skin appearance. Interestingly, the wound area was significantly declined on days 3, 6, 9, 12, 15 and 18 PW than control group (Fig. 1D). These wound closure rates made a good match to the results of H&E staining. Thus, licorice extract significantly contributed to wound healing compared to the control untreated group.

3.4. Leukogram, immunological and antioxidant parameters

At the 18th DPW, leukogram findings revealed that, total leucocytic count (TLC), lymphocyte, neutrophil and monocyte counts were significantly elevated in both licorice treated groups compared with control group as shown in Fig. 3A. Moreover, the phagocytic activity and phagocytic index of neutrophils as portrayed in Fig. 3A revealed a significant enhancement in both topical and oral licorice-treated groups more than those of the control group. Interestingly, a significant increase in SOD, GPx activities and GSH content with a notable reduction in the MDA levels were observed in licorice extract-treated groups compared with control untreated group (Fig. 3B).

3.5. Histological evaluation of healed wounds

Eighteen DPW, the untreated wounds in the control group (CG) revealed delayed healing activity with minimal re-epithelialization where there were one to two layers of epithelial epidermal cells formed under scab. In few sections, the wounds were not yet completely bridged by new layers of epithelial cells (Fig. 4a) which were, however, positive for keratin 14. In addition, the dermis was only lightly infiltrated with macrophages and very few neutrophils and the granulation tissue was rich in fibroblasts and newly formed blood vessels (Fig. 4b and c). Moreover, collagen fibers were characterized by poor orientation and disorganization. On the other hand, epidermal layers of wounds from licorice-treated rats (topical and oral) were thicker than those in wounds from the control group. In addition, the wounds were completely re-epithelialized and necrotic tissue was completely replaced with granulation tissue with decreasing cellularity and with an increasing presence of collagen (Fig. 4d and g). Moreover in the examined sections, complete differentiation process of keratinocytes was confirmed by the normal process of keratinization over the entire wound (Fig. 4d and g). Both topical and oral licorice groups demonstrated similar wound healing activity. However, topical-treated group showed excessive re-epithelialization and thickening of the epidermis as well as marked inflammatory cells infiltrations (Fig. 4d and e) in the dermis predominately macrophages, very few neutrophils and lymphocytes (Fig. 4f) and hair follicles regeneration. The tissue sections from the oral-treated group showed complete re-epithelialization, minimal inflammatory cells infiltration mostly macrophages, very few neutrophils and lymphocytes, and maximum maturation of granulation tissue with well-organized accumulation of collagen fibers and significant increase in the number of fibroblasts in the dermis in the wounded area as well as more effective re-organization of the dermis compared to the control group (Fig. 4g–i).

3.6. Immuno-expression of α-SMA, PDGFR-α, FGFR1 and cytokeratin 14 on skin wound and scoring of immune-reactivity

Regarding immune-reactivity in all experimental groups, α-SMA showed cytoplasmic expression in myofibroblasts which were already present at the regeneration phase of wound healing in the dermis and subcutis as well as endothelial cells of new blood vessels and very few of the basal keratinocytes of the epidermis. As shown in Fig. 5A, the control group showed few numbers of α-SMA-positive myofibroblasts (arrow) in the dermis, where topically-treated group showed numerous α-SMA-positive myofibroblasts together with few mmunoreactive basal cells

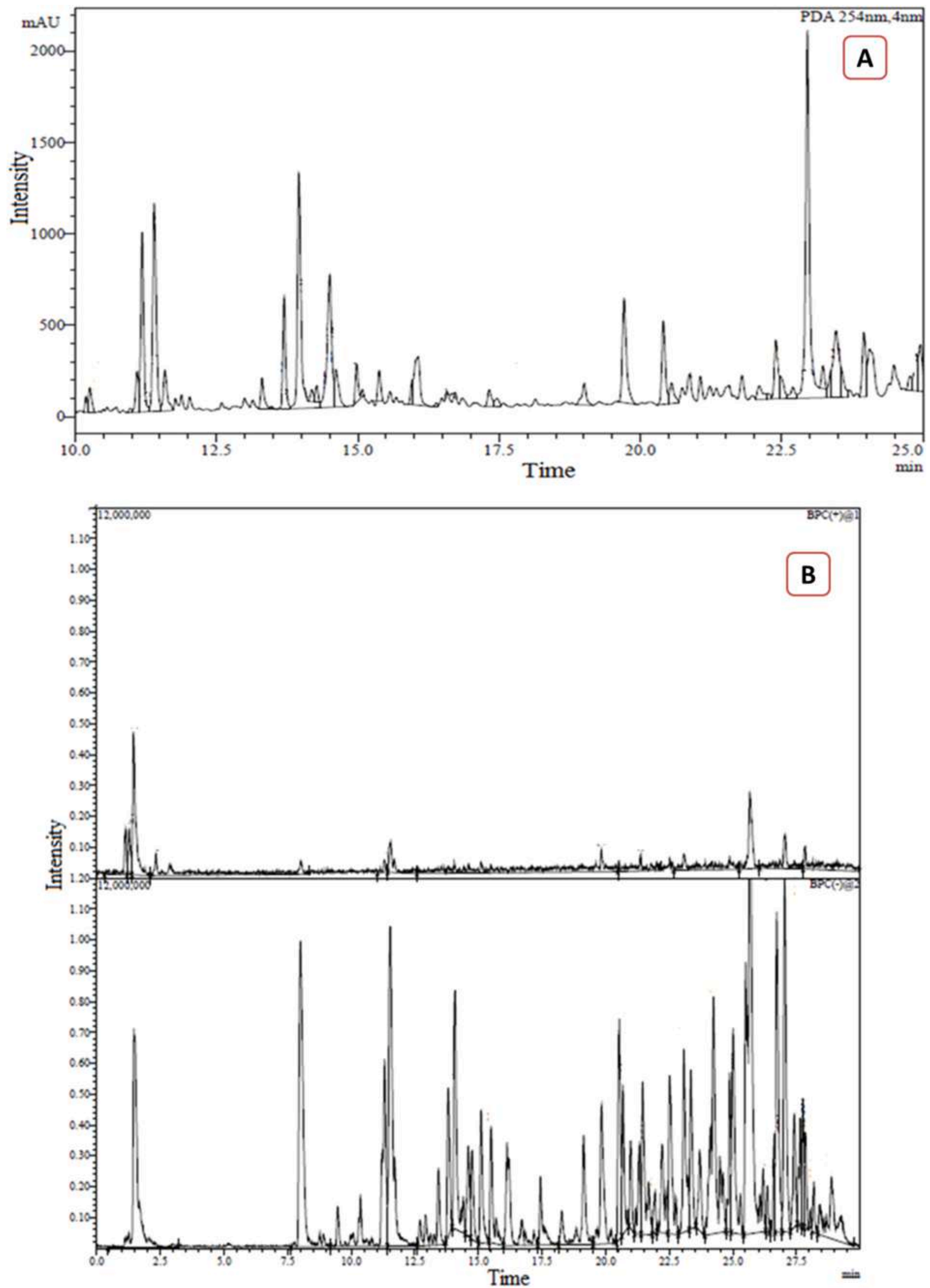

Fig. 2. (A)UPLC-PDA spectrum of licorice extract. (B) UPLC-MS total ion chromatograms of licorice extract in ESI+ (top) and ESI- (bottom) modes.

Table 2
UPLC-PDA-MS/MS results for 70% alcohol extract of the commercially available licorice.

NO.	R_t min	$[M-H]^-$ m/z	$[M+H]^+$	MS^2 ions m/z	Identification
1	7.69	431		161, 155, 125, 97	Licoagroside B
2	8.00	431		137, 113	Unknown
3	8.01		595	287	Kaempferol-3-O-rutinoside
4	8.21	431		137, 113	Unknown
5	8.36	209		165, 135, 121, 119, 93	Hydroxybenzyl malonic acid (HBMA)
6	8.15	563		563, 546, 517, 473, 443, 395, 383, 353, 325, 296, 283, 261, 223, 191, 175, 152, 113, 89	Isoshaftoside
7	8.37	563	565	563, 545, 503, 473, 443, 395, 383, 353, 311	Shaftoside
8	8.67	577	579	577, 473, 457, 425, 413, 383, 353, 311	Isoviolanthin
9	8.98	577		577, 485, 457, 395, 383, 353, 325, 297, 283, 137, 111	Violanthin
10	8.96	549		549, 429, 255, 135, 109	Liquiritin apioside
11	9.04	549		549, 255, 135, 119	Liquiritin apioside isomer
12	9.16	417		255, 148, 135, 119	Liquiritin
13	9.27	417		255, 135, 119	Liquiritin isomer
14	9.58	565		271, 177, 151, 119	Hydroxyisoliquiritin apioside
15	9.75	565		565, 271, 227, 203, 164, 151, 119	Hydroxyisoliquiritin apioside isomer
16	10.15	549		255, 135, 119	Isoliquiritin apioside
17	10.25	549		255, 135, 119	Isoliquiritin apioside isomer
18	10.25		563	269	Glycyroside
19	10.51	565		309, 271, 135, 121	Butein-4-O—glucopyranosyl-apiofuranoside
20	10.44	695		549, 531, 429, 351, 255, 163, 145, 119	Licorice glycoside D2/D1
21	10.57	695		549, 531, 399, 255, 163, 145, 119	Licorice glycoside D2/D1
22	10.58	417		254, 225, 213, 148, 135, 119	Isoliquiritin
23	10.70	417		255, 135, 119	Isoliquiritin isomer
24	11.21	692		549, 531, 255, 160, 135	Licorice glycoside E
25	12.10	692		549, 334, 297, 255, 160, 116	Licorice glycoside E isomer
26	11.42	285		285, 270, 177, 150, 120	Licochalcone B
27	11.82	285		285, 253, 241, 225, 217, 211, 198, 175, 161, 149, 136, 108	Unknown
28	11.97	255	257	135, 119, 91	Isoliquiritigenin
29	12.66	255		255, 237, 213, 209, 175, 168, 145, 135, 119, 109, 91	Pinocembrin
30	13.06	269		269, 237, 209, 175, 161, 149, 133, 120, 92	Echinatin
31	13.18	269	271	269, 254, 237, 225, 210, 197, 151, 133, 117	Echinatin isomer
32	13.72		453	ND	Unknown
33	13.76	821		759, 610, 469, 351, 307, 193, 103	Glycyrrhizin
34	14.51	821		821, 351, 193	Glycyrrhizin isome
35	14.32	255	257	255, 135, 119, 91	Isoliquiritigenin isomer
36	14.94	255		255, 240, 212, 197, 180, 151, 135, 121, 119, 105, 91	Isoliquiritigenin isomer
37	14.71	267	269	267, 252, 223	Formononetin
38	15.24	267		267, 252, 223, 195, 163	Isoformononetin
39	15.06	369	371	369, 337, 285, 229, 177, 150, 124	Prenylated flavonoid
40	15.49	369		369, 354, 351, 337, 321, 308, 297, 285, 259	Prenylated flavonoid
41	15.12	339		311, 242, 201, 173, 164, 149, 136, 109	Unknown
42	15.45	339		177, 161, 133, 122, 109	Esculin
43	15.39	357		235, 217, 177, 161, 123	Hydroxy dihydroesculin
44	15.68	357		321, 311, 245, 235, 217, 195, 177, 161, 151, 135, 121, 105	Hydroxy-dihydroesculin isomer
45	15.70	341		167, 149, 137, 121, 109	Unknown
46	15.75		323	ND	Unknown
47	15.52	341		235, 216, 205, 189, 177, 161, 135, 122	Dihydroesculin
48	15.87		233	ND	Unknown
49	16.04	335		335, 307, 291, 263	Isoglabrone
50	16.37		355	ND	Unknown
51	16.94	335		335, 307, 291, 263, 247, 231, 213, 201,199,161,136, 109	Glabrone
52	16.90	321	323	321, 306, 291, 277, 266, 261, 227, 213	Glabrene
53	17.20	321		322, 306, 291, 278, 266, 251, 244, 227, 212, 199, 193, 175, 155, 146, 133, 105	Isoglabrene
54	17.21		337	ND	Unknown
55	16.91	339		321, 281, 266, 253, 237, 213, 187, 171, 159, 145, 135	Benzoic acid derivative
56	17.09	339		293, 229, 167, 137, 123, 109	Unknown
57	17.26	367	369	367, 353, 309, 298, 265, 243, 218, 190, 148	Glycycoumarin
58	18.09		355	ND	Unknown
59	18.32	367		367, 349, 323, 305, 213, 201, 179, 165, 147, 135, 121, 109	Unknown
60	17.10	339		293, 229, 167, 137, 123, 109,	Unknown
61	18.11	339		203, 159, 135	5-Prenylbutein
62	18.46		409	ND	Unknown
63	18.71	323		323, 213, 201, 187, 175, 135, 107	Glabridin
64	19.56	323		203, 201, 159, 119	Isobavachalcone
65	18.45	425		221, 203, 177, 148	Methyl kanzolol Y
66	19.22	425		235, 217, 205, 177, 174, 136	2́,4́,6́,3,4-pentahydroxy-3́,5-diprenyl dihydrochalcone
67	19.46	409		235, 217, 205, 177, 161, 135, 122	Kanzolol Y isomer
68	20.22	409		363, 235, 217, 189, 177, 161, 148	Kanzolol Y
69	19.64	391		203, 187, 159, 132	Glabrol
70	20.16	391		391, 306, 203, 187, 175, 157, 132	Hispaglabridin A

(continued on next page)

Table 2 (*continued*)

NO.	R_t min	[M − H]⁻m/z	[M + H]⁺	MS² ions m/z	Identification
71	19.68	423		219, 203, 149	Unknown
72	20.31	423		229, 193, 174, 149, 125	Gancaonin E
73	20.34	393	395	375, 349, 324, 215, 203, 189, 177	Kanzonol x
74	20.08	351		351, 336, 323, 308, 283, 271, 201, 175, 149, 131, 107	Coumarin derivative
75	20.69	351		351, 281, 201, 187, 175, 163, 149, 137, 107	Coumarin derivative
76	21.50	659		335, 201, 135, 109	Glabrone+glabridin
77	21.53		627	303	Quercetin-3-sophoroside
78	21.60	659		659, 523, 471, 349, 335, 324, 279, 201, 148, 135	Glabrone+glabridin isomer
79	22.01	337		337, 322, 279, 213, 201, 187, 175, 149, 123, 107	4́-O-Methylglabridin
80	23.04	407		203159, 148	3-Hydroxy glabrol
81	23.72	407		245, 229, 219, 201, 185, 177, 161, 146, 130, 106	Glyinflanin A
82	24.29	657		639, 481, 455, 323, 267, 187, 175, 135	Glabridin+sophoracoumestan A
83	25.35	657		333, 324, 201, 135, 109	Glabridin+sophoracoumestan A isomer
84	25.61	405		203, 184	Unknown
85	25.81	405		387, 363, 330, 305, 219, 203, 185, 169, 145, 117	Gancaonin Q
86	26.11	673		655, 495, 387, 349, 335, 323, 253, 214, 201, 177, 147, 121	Flavonoid dimers
87	26.15	673		495, 437, 349, 335, 323, 308, 281, 177, 159, 135	Flavonoid dimers
88	26.43	419		419, 391, 349, 331, 278, 267, 243, 231, 201, 175, 161, 147, 107	Kanzonol F
89	26.59	419		419, 283, 243, 231, 201, 175, 161, 145	Unknown
90	26.79	455		455, 225	11-Deoxyglycyrrhetinic
91	26.88	455		455	11-Deoxyglycyrrhetinic isomer
92	27.14	389		389, 201, 187, 132	Hispaglabridin B
93	27.42	389		389, 201, 187, 179, 132	Hispaglabridin B
94	27.03	353		353, 347, 323, 214, 201, 175, 135, 121	Unknown
95	27.33	353		323, 201, 135	Unknown
96	27.28	475		271, 203, 159	Glabrol derivative
97	27.32	475		271, 203, 184, 161	Glabrol derivative
98	28.06	271		271, 254, 225, 223	Unknown
99	28.38	271		271, 227, 225, 224	Unknown
100	28.39	617		617, 161, 135	Unknown
101	28.44	617		618, 617	Unknown

(head arrow). Orally-treated group showed numerous α-SMA positive myofibroblasts (arrow).

While PDGFR-α showed cytoplasmic expression in fibroblast, endothelial cells of blood vessels, hair follicles cells and some of the inflammatory cells predominantly macrophage and lymphocytes in the dermis as well as proliferating keratinocytes of the epidermis. In Fig. 5B, control group showed few numbers of PDGFR-α mmunoreactive cells in the dermis including fibroblasts (head arrow), inflammatory cells predominantly macrophages (fine arrow), endothelial cells of blood capillaries (thick arrow) and keratinocytes. Topically-treated group showed moderate numbers of PDGFR-α-positive cells mainly fibroblasts (head arrow) together with epidermal epithelial cells while orally-treated group showed numerous PDGFR-α-positive cells mainly fibroblasts (head arrow) as well as keratinocytes.

In addition, FGFR1 showed cytoplasmic expression in fibroblast, few of the inflammatory cells predominantly macrophage and the proliferating keratinocytes of the epidermis. In Fig. 5C, control group showed few numbers of FGFR1-positive cells in the dermis and keratinocytes. Topically-treated group showed large numbers of FGFR1-positive cells in the dermis and keratinocytes while orally-treated group showed moderate numbers of FGFR1-positive cells in the dermis.

In Fig. 5D, control group showed incomplete or thin epidermal layers (white lines). While both oral and topical groups showed increase of Cytokeratin 14 expression by keratinocytes. There is increase in staining intensity and epidermal thickness in samples collected from oral and topical treatments with licorice.

As seen in Table3 and Fig. 5 on the 18th DPW, the immune-expression of α-SMA, was significantly increased in both licorice topical and oral treated groups (53.83 ± 2.82 and 56.08 ± 2.47 respectively) compared with that in the control group, which was 24.67 ± 6.70 (Fig. 5A).

Moreover, immunoreactivity of PDGFR-α was significantly elevated in topical and oral supplied groups (72.75 ± 1.59 and 93.33 ± 0.8 respectively) compared to those in the control group which was 65.00 ± 2.89 (Fig. 5B). Although FGFR1 was markedly increased in both oral-treated group and topical-treated group, consistent with the complete healing process in the wound. Furthermore, the immune-reactivity of FGFR1 in both licorice-treated groups was much higher than those in the control group (93.33 ± 0.83 and 58.20 ± 5.16 respectively) (Fig. 5C). Moreover, the immune-reactivity of Cytokeratin 14 in rats groups treated with licorice either topically or orally was prominently much expressed (Fig. 5D) compared with control untreated group.

3.7. Gene expression analysis

Regarding the impact of licorice alcoholic extract on the molecular mechanisms involved in wound healing process we focused on angiogenic genes as bFGF, VEGF and TGF-β as shown in Fig. 3C. The relative expression levels of bFGF, VEGF and TGF-β genes were enhanced in the groups supplied with licorice in comparison with the control untreated group. Interestingly, the relative expression levels of bFGF, VEGF and TGF-β genes were strictly enhanced in topically-applied group than the orally-administered group compared with the control group.

4. Discussion

Wound repairing is an orchestrated process with many dynamic phases to restore the cellular structure of the damaged tissues back to its initial healthy status. It involves three overlapping phases: the inflammatory phase which represents the established homeostasis and inflammation; then the proliferative phase which includes epithelialization, fibroplasia, angiogenesis, and formation of granulation tissue and finally the remodeling phase that includes collagen deposition and small scaring tissue formation which ultimately determines the strength and appearance of the healed area [52,53]. Although, licorice wound healing efficacy is already reported, little is known about the mechanism associated with its action. Here we explored its wound healing potential in rats through a series of indices related to immune-hematological, antioxidant, histopathological and IHC assessment as well as the molecular gene expressions targeting angiogenesis. We detected that

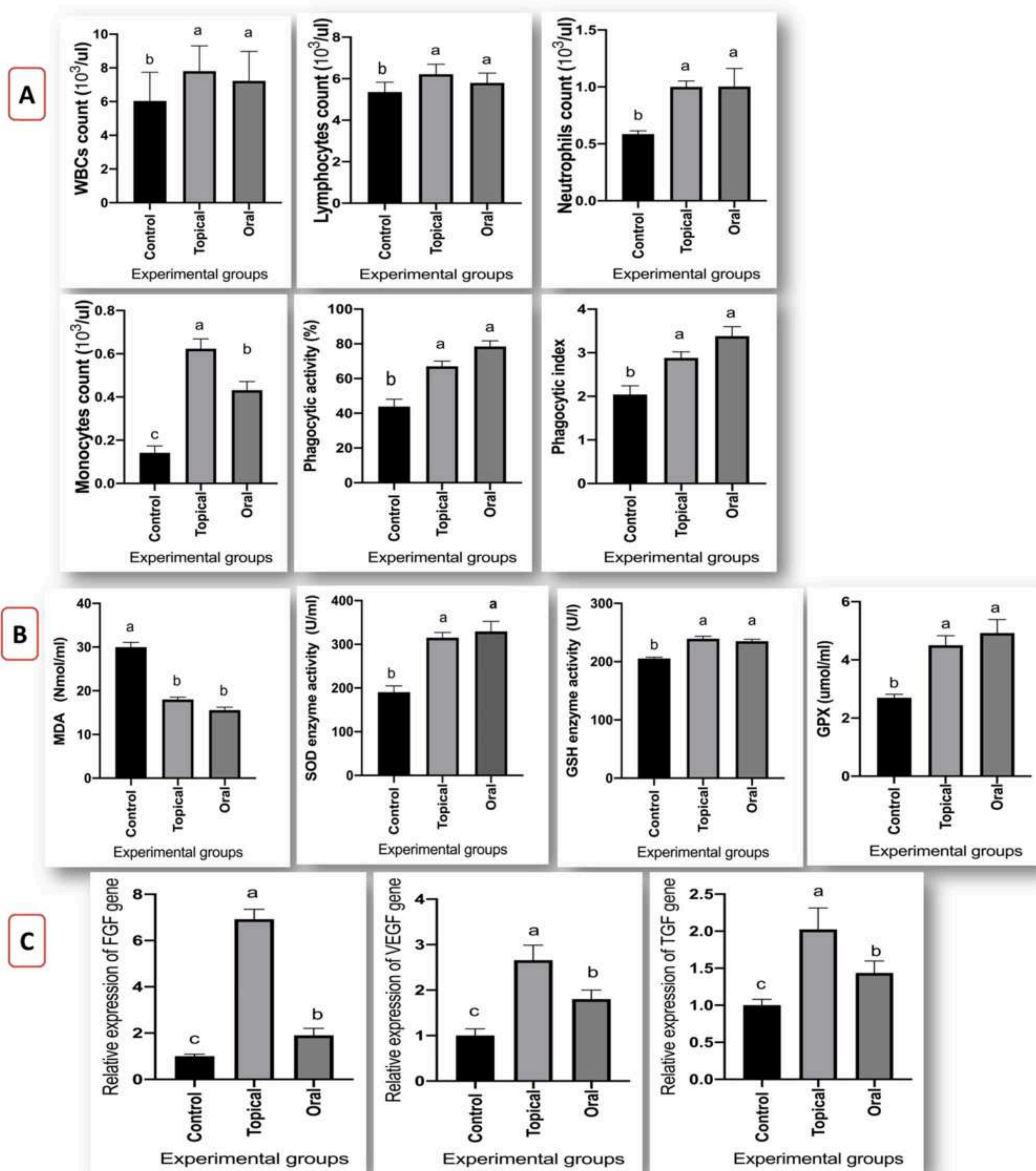

Fig. 3. (A) Leukogram findings of total leucocytic count (TLC), lymphocyte, neutrophil and monocyte counts, Phagocytic activity and phagocytic index of neutrophils at the 18 day post wounding in licorice treated groups compared with control group. (B) Oxidative stress and antioxidant parameters at 18th day post wounding in licorice treated groups compared with control group. Malondialdehyde (MDA), Superoxide dismutase (SOD), Reduced glutathione (GSH) and Glutathione peroxidase (GPx). (C) Relative gene expression of basic fibroblast growth factor *(bFGF)*, Vascular endothelial growth factor *(VEGF)* and Transforming growth factor *(TGF-Β1)* genes in wounded rat skin of control group, topical and oral treated rat with liquorice alcoholic extract. Values are expressed as mean ± SE from triplicate groups (n = 8). Bars with a,b,c letters are significantly different from each other ($P < 0.05$).

topical or oral application of licorice extract enhanced rats immune response through a significant increase in total and differential leucocyte counts, phagocytic activity and phagocytic index of neutrophils compared with the control untreated group. Similarly, Wilgus et al. [54] who documented the central role of aseptic wound condition provided by phagocytosis and bacterial killing action of neutrophils to accelerate wound healing process. Gupta et al. [55] and Wang et al. [56] linked *G. glabra* antimicrobial properties particularly on *Staph. aureus*, *Esch. coli*, *Pseudo. aeruginosa*, *C. albicans*, and *Bacillus subtilis* to its rich content with saponins, alkaloids and flavonoids as well as glabridin, glabrol,

A Photomicrograph of histopathological changes in rat skin tissue

B Thickness of the epidermis in different treatment groups

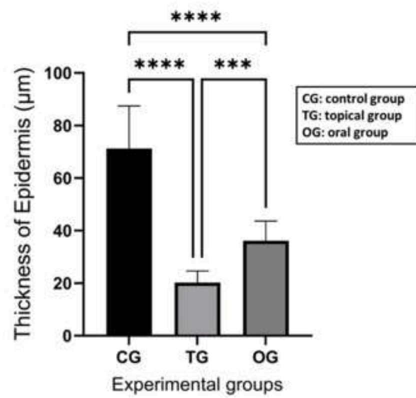

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Fig. 4. (A) photomicrograph of histopathological changes in rat skin tissue 18 days after surgical wound from three experimental groups which include (a, b, c) control (CG), (d, e, f) topical licorice application (TG), and (g, h, i) oral licorice administration (OG). (a) showing incomplete epithelialization and beginning of epidermal cell proliferation and migration (new epidermis, E) beneath scab. (b) showing granulation tissue rich in fibroblasts and newly formed blood vessels. (c) showing poor oriented and disorganized collagen fibers together with few inflammatory cells predominantly macrophages (arrow head). (d) showing complete epithelialization over the wounded area as well as presence of keratin (arrow head) over the epithelium exhibiting successful differentiation of keratinocytes. (e) showing excessive epithelialization and marked inflammatory cells infiltration in the dermis. (f) showing marked inflammatory cells (arrow head) infiltration in the dermis predominately macrophages. (g) showing complete re-epithelialization. (h) showing maturation of granulation tissue with decreasing cellularity and with an increasing presence of collagen. (i) showing well-organized accumulation of collagen fibers and very few inflammatory cells (arrow head) in the granulation tissue. H&E. X 100, 200, 400. (B) Thickness of the epidermis in different treatment groups, Values are expressed as mean ± SD from triplicate groups (n = 8). Bars with asterisks are significantly different from each other ($P < 0.0001$).

glabrene, hispaglabridin A, hispaglabridin B, 40–methylglabridin, and 3–hydroxyglabrol. They attributed licorice antibacterial activity to the declined bacterial gene expression, bacteria growth prevention and to the reduced bacterial toxin secretion. Macrophages and monocytes are considered critical cells of the inflammatory phase in wound healing [57]. These cells participate in microbial phagocytosis, cellular debris and damaged matrix removal by secretion of matrix metallo-proteinases. In addition to their role as a main source of cytokines and growth factors to stimulate the proliferation of fibroblasts and enhance collagen biosynthesis [58]. In accordance with numerous studies which confirmed the essential role of macrophages for proper skin wound healing [59,60]. In line with Shinada et al. [61] who mentioned that licorice root administration elevated leukocyte infiltration, blood flow and effectively reduced inflammation via steroidal, cortisone-like actions. Moreover, Soufy et al. [62] and Okda et al. [63] attributed the immune-stimulatory effect of licorice to Glycyrrhetinic acid content which enhances the anti-inflammatory cytokines production, especially IL-7 and IL-10 and inhibits secretion of pro-inflammatory cytokines, especially TNF-α and IL-6 [64,65].

Interestingly, in the present study we demonstrated that licorice applications enhanced antioxidant status and notably declined the oxidative stress biomarker compared with the control untreated group. Shenoy et al. [66] and Castangia et al. [67] attributed the accelerated wound healing to licorice-isolated sterols, polyphenols specifically Glycyrrhizic acid. Although licorice enhanced wound contraction, increased the epithelization rate, decreased cell necrosis, improved vascularity and reduced skin damages via its free radical scavenging and antioxidant potentials [68]. Moreover, Veratti et al. [69] documented the inhibition of human keratinocytes DNA damage induced by UV radiation by 18β-glycyrrhetinic acid and glabridin treatment. The major reason for licorice application choice is its efficient antioxidant properties specifically duo to phenolic content [70], flavonoids [23], sterols and polyphenols [66,67], isoflavones [71]. In the same line, licorice content of licochalcones B and D also exhibited a strong scavenging activity on DPPH that protected fibroblasts against oxidative stress through its ability to prevent the microsomal lipid peroxidation [72].

The wound closure occurs during the proliferative phase as a consequence of wound contraction by wound edges movement toward the center [73]. The progression of wound healing can be judged by periodic assessment of the contraction of the excised wound [74]. Here the accelerated wound healing was evidenced by declined wound closure time as a result of better wound contraction in licorice-treated groups than the control group. We noticed that licorice treated rats did not trigger any signs of biting or scratching or restlessness reflecting that licorice extract did not show any pain or irritation signs supported by histopathological examination of licorice-treated groups that showed complete re-epithelialization and replacement of necrotic tissue with granulation tissue as well as reduced cellularity and increased collagen deposition. Zaki et al. [75] reported faster contraction of the healed wound topically treated with licorice extract compared with untreated ones or those subjected to topical application of the vehicle eucerin in rabbits. As wound contraction is mediated by generation of cellular forces in the contractile elements of fibroblasts and myofibroblast due to the expression of α-SMA in microfilament bundles [73]. In accordance with our IHC expression for α-SMA which was significantly enhanced in licorice treated groups compared with the control group. Midwood et al. [76] mentioned that the initial minimal degree of wound contraction during this early stage is related mainly to the activity of the fibroblast in the wound edges and subcutaneous tissue. On the same context, Oloumi et al. [77] revealed that the number of fibroblasts and capillary buds were significantly higher in the licorice-treatment groups while the epithelial gap was much less reflecting better re-epithelialization than the control group. Hanafi et al. [78] reported that licorice creams (5% and 10% w/w) significantly increased the epidermal formation, collagen deposition and neovascularization in comparison to the control group in full-thickness wound healing in Guinea pigs. Castangia et al. [67]; Kotian et al. [79] linked licorice promoted wound healing, accelerated epithelization, ameliorated remodeling and efficient reduction of atopic dermatitis (AD) symptoms to saponins, sterols and polyphenols free radical scavenging and antioxidant activities which are known to reduce lipid peroxidation, thereby reduce cell necrosis and improve vascularity.

There is an important interaction between keratinocytes and fibroblasts especially in the mid and late phase of wound healing [80,81]. It is noteworthy that fibroblasts express both isoforms of the PDGF receptor; PDGFR-α and PDGFR-β [82]. PDGF enhances fibroblasts proliferation as well as production of extracellular matrix (ECM) by these cells. These reports confirmed the critical roles of PDGF, FGF and their receptors for wound healing. Our observations revealed more monocyte counts with higher IHC expression of bFGF, TGF-β1, and PDGFR1, FGFR1 with complete epithelialization and faster wound healing in licorice-supplied groups than the defectively epithelialized control group. Interestingly, licorice supplied topical and oral groups were side by side in all the evaluated parameters to be more efficient in wound healing than the untreated control group but at IHC and molecular gene expression analysis, the licorice topical application reflected higher response than the oral application except for PDGFR1 which was higher in oral group than the topical group but in all situations both licorice-supplied groups were significantly more efficient for accelerating wound healing than the control group. Our results are in accordance with Najeeb and Al-Refai [31] who detected that the topically applied licorice extract exhibited strong anti-inflammatory activity by inhibiting nitric oxide and prostaglandin E2 production by macrophages. Licorice extract also reduced the release of pro-inflammatory cytokines and CD14, tumor necrosis factor and interleukin-6 (IL-6), and elevated IL-10 production [83,84]. Furthermore, licorice contains vitamin E, B complex, pantothenic acid, lecithin, biotin, niacin, manganese, calcium, calcium salts, proteins, and nucleic acids which may aid in wound healing [85]. Similarly, Fuller [86] who applied 1% and 2% licorice topical gel and found its efficacy in reducing erythema, edema and itching after two weeks. Moreover, fibroblasts can acquire a myofibroblast phenotype under the control of keratinocytes. During epidermis regeneration, activated keratinocytes produced specific keratin proteins which reflected the level of their differentiation [87]. Among these proteins, keratin 14 has been considered as a marker of the basal epidermal layer [88]. Moreover, Pastar et al. [89] implied that topical application of basal keratinocytes on wound bed-induced keratinocyte proliferation factors including keratin 5, 14 that promoted reorganization of the underlying epidermal and dermal layers. Although, declined K14 expression has been associated with defective re-epithelialization [90]. In accordance with our study, proliferation marker K14 expression was

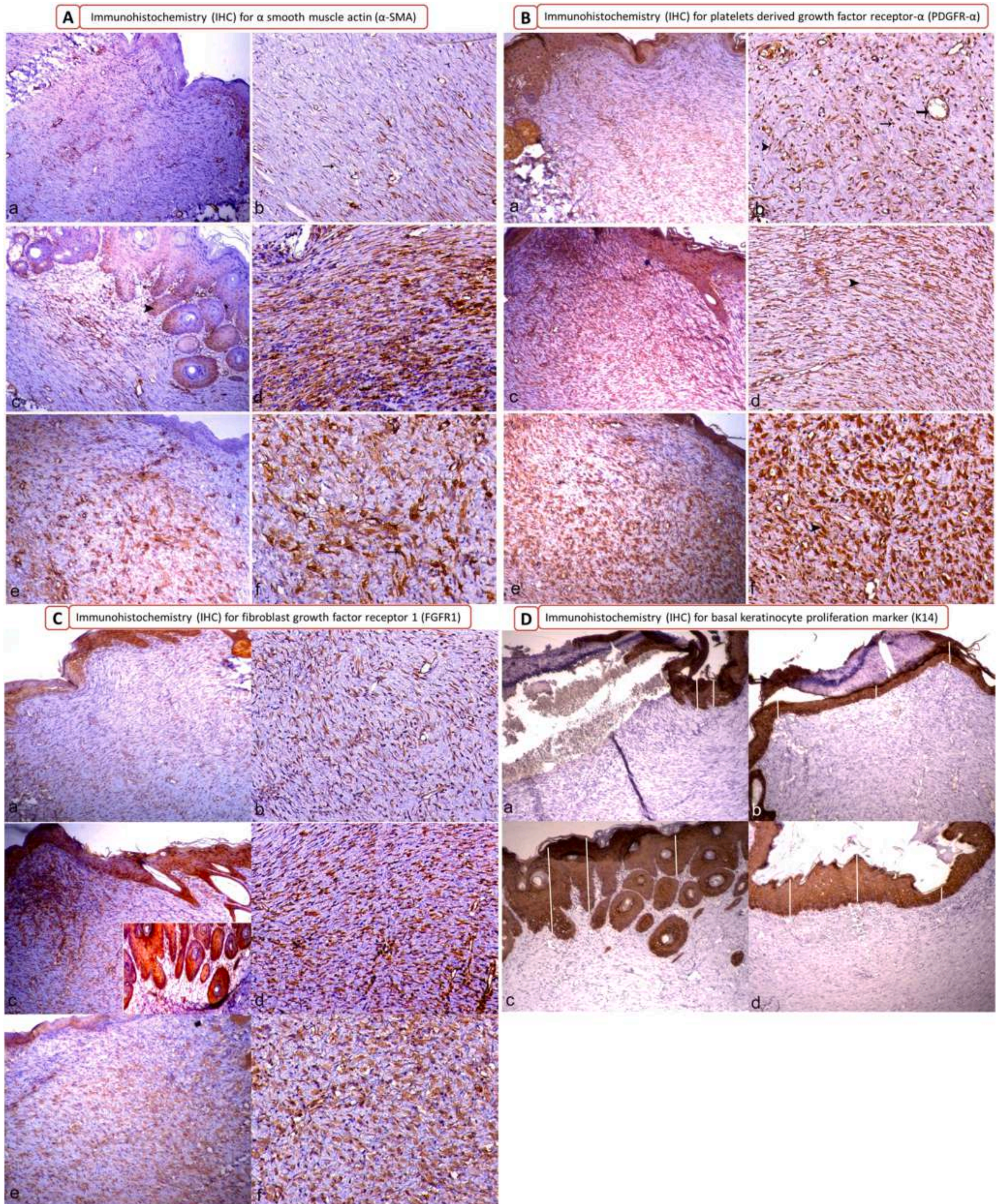

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Fig. 5. (A) Immunohistochemistry (IHC) for α smooth muscle actin (α-SMA)-positive myofibroblasts in skin wounds on day 18 post wounding. Histological sections were stained with an anti α-SMA (marker for myofibroblast) antibody and counterstained with Mayer's haematoxylin. (a-b) Control group, (c-d) Topical treated group, (e-f) Oral treated group. (B) Immunohistochemistry for platelets derived growth factor receptor-α (PDGFR-α) positive cells specially fibroblasts in skin wounds on day 18 post wounding. Histological sections were stained with an anti PDGFR-α antibody and counterstained with Mayer's haematoxylin. (a-b) Control group, (c-d) Topical treated group, (e-f) Oral treated group. (C) Immunohistochemistry for fibroblast growth factor receptor 1 (FGFR1) positive cells specially fibroblasts and keratinocytes in skin wounds on day 18 post wounding. Histological sections were stained with an anti FGFR1antibody and counterstained with Mayer's haematoxylin. (a-b) Control group, (c-d) Topical treated group, (e-f) Oral treated group, showing moderate numbers of FGFR1 positive cells in the dermis. 100,200X magnification. (D) Immunohistochemistry of the skin tissue from the wound site on day 18 to determine the expression of basal keratinocyte proliferation marker (K14). Histological sections were stained by immunohistochemistry with an anti-Cytokeratin 14 antibodies and were counterstained with Mayer's haematoxylin. (a-b) control group showing incomplete or thin epidermal layers (white lines). (c-d) showing increase of Cytokeratin 14 expression by keratinocytes from (c) topical and (d) oral treatments with licorice. There is increase in staining intensity and epidermal thickness in samples collected from oral and topical treatments with licorice. 100X magnification.

Table 3
Immunohistochemical analysis and Scoring of immune-reactivity.

	Control gr.	Topical gr.	Oral gr.
(A) Positive area of α-SMA	24.67 ± 6.70^{b}	53.83 ± 2.82^{a}	56.08 ± 2.47^{a}
(B) positive area of PDGFR-α	65.00 ± 2.89^{c}	72.75 ± 1.59^{b}	93.33 ± 0.83^{a}
(C) positive area of FGFR1	18.67 ± 2.60^{c}	70.20 ± 4.31^{a}	58.20 ± 5.16^{b}

Mean values with different letters at the same row differ significantly at $P < 0.0001$.

much increased in licorice-treated groups compared with the control group. As far as we know, this is the first report confirming that inducible FGFR1 (BFGFR), PDGFR-α and α-SMA expressions are sensitive to licorice administration and licorice harnessed myofibroblasts differentiation to accelerate dermal wound contraction and closure. We suggest that licorice with its rich components recruited and activated macrophages and fibroblast activities to produce growth factors that enhanced the fibroblast proliferation, collagen deposition and angiogenesis as crucial factors for the healing process [91].

In the current study, we hypothesized that the presence of licorice extract possibly enhanced TGF-β availability in the extravascular environment for priming cells thus enhanced their response to the normal regulatory factors at the site of injury. In this study, we found marked gene expression levels for bFGF, VEGF and TGF-B in licorice-treated groups compared with the control untreated group. Angiogenesis is a process where the blood circulation in the area of the wound is increased. This improves the transport of nutrients and oxygen, which are crucial for the healing process and re-epithelialization of the wound site [92]. On the same context, Elbialy et al. [12] linked the marked gene expression levels of bFGF and VEGF to the remarkable wound healing process by enhancing angiogenesis and collagen deposition thus improved the epithelialization rate. On the same line, Ortega et al. [93] reported that FGF signaling has a critical role for efficient healing, while the bFGF deficient mice reflected a state of wound healing impairment. Species chemical markers for licorice were proposed by Scalabrin [32], Farag et al. [34] and Rizzato et al. [21]. They identified glabridin, glabrol, 3-hydroxyglabrol, kanzonol Y as markers for *G. glabra*; glycycoumarin for *G. uralensis* and licochalcone A and glyinflanin A for *G. inflata*. Our UPLC-PDA-MS/MS results indicated the detection of these markers in the extract of the licorice sample used in this study (Table 2) confirmed that three species namely *G. glabra, G. uralensis* and *G. inflata* mediated the observed biological effects in this work. Apart from these markers, these three species have several compounds in common as previously mentioned in the results section. The extract was found rich in flavonoids, chalcones, coumarins, phenolic compounds and saponin (mainly glycyrrhizic acid) which are excellent candidates for anti-inflammatory and antioxidant effects. Glabridin, glycyrrhizic acid and licochalcone A, showed an anti-inflammatory effect also these compounds in addition to hispaglabridin A and B exhibited antioxidant activities via several mechanisms [94]. However, further investigation on the regulatory role of licorice active principle on the expression of several profibrotic genes and matrix metalloproteinases (MMPs) is needed.

5. Conclusion

Licorice extract positively accelerated cutaneous wound healing in rats model through exhibiting potent antioxidant and free radical scavenging properties therefore modulated histopathological morphologic alteration, enhanced wound angiogenesis and collagen deposition via up regulating angiogenic bFGF,VEGF and TGF-B genes. More specifically, we demonstrated for the first time that inducible FGFR1 (BFGFR), PDGFR-α and α-SMA expression are sensitive to licorice administration providing a potential cellular and molecular basis for future treatments with a promising natural and biological active source which can positively influence the cutaneous wound healing via numerous bioactive compounds namely saponins, flavonoids and chalcones of licorice.

Institutional review board statement

The study was conducted based on the recommended NIH Guide for the care and use of laboratory animals by the Faculty of Veterinary Medicine Ethics Committee, Kafrelsheik University, Egypt. All precautions were followed to diminish animal suffering during the experiment.

Informed consent statement

Not applicable.

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CRediT authorship contribution statement

Doaa Hosny Assar: Conceptualization, Data curation, Investigation, Writing – original draft. **Zizy Ibrahim Elbialy:** Data curation, Formal analysis, Writing – review & editing. **Nagwan Elhabashi:** Formal analysis, Methodology. **Amany Ragab:** Formal analysis, Methodology, Resources. **Sally Rizk:** Investigation, Methodology. **Ayman Atiba**: Methodology, Validation. **Abd-Allah Mokhbatly:** Project administration, Validation. **Soad Al Jaouni:** Project administration, Resources. **Aishah E. Albalawi:** Writing – review & editing. **Norah A. Althobaiti:** Writing – review & editing.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its Supplementary materials.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112151.

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