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RESEARCH ARTICLE

Anchusa officinalis accelerates wound healing via the improvement of transforming growth factor beta 1 expressions, antioxidant levels, and inhibition of TNF-α, IL-6

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ABSTRACT

Wound healing is an intricate, complicated process that needs special attention because of its related complications that may occur if not treated properly or because of therapeutic insufficiency. Common bugloss (*Anchusa officinalis* L.) is a deep-rooted, hairy perennial herb used in folk medicine for numerous human issues, including wound recovery. To delineate its safety and healing potentials, we investigated the acute toxicity and wound-healing effects of *Anchusa officinalis* L. (APEAO) aerial part extracts on excisional neck injury in rats. A uniform dorsal neck cut was formed in twenty-four albino rats, which were arbitrarily divided into 4 groups and treated daily with a topical 0.2 ml dose of the following: group A, rats received 10% tween 20; group B, rats received intrasite gel; groups C and D, rats had 250 and 500 mg/kg of APEAO, respectively. The APEAO treatment did not cause toxic damage in rats administered with up to 5 g/kg APEAO. In the wound experiment, APEAO-treated skin exhibited significantly higher deposition of tissue collagen and fibroblast cells. In contrast, inflammatory cells were significantly lower in the recovered tissues of than positve control rats. Topical application of APEAO caused positive modulation of Transforming Growth Factor Beta 1 (angiogenesis) in recovered skin, indicating elevated tissue growth and faster wound-healing action. Moreover, APEAO treatment caused a significant elevation in tissue antioxidants (Superoxide dismutase, glutathione peroxidase, and catalyze) and hydroxyproline (collagen) content, lowering Malondialdehyde levels compared to vehicle rats. Serum inflammatory chemicals (Transforming growth factor α, Interlukin-6, and Interlukin-10) were significantly modulated following APEAO application. The outcomes revealed significant tissue regeneration potentials of APEAO exhibited by its modulatory actions on several cellular processes, which could serve as scientific evidence for future investigation regarding the production of potent pharmaceuticals for faster wound contraction.

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Anchusa officinalis; wound-healing; antioxidants; immunohistochemical; inflammatory cytokines

1. Introduction

Wound caring can be an expensive procedure, taking a large part from country budgets, considering its rapid global marketing of $12 billion in 2020 and its expectancy to reach $35 billion by 2027 based on recent statistics for chronic wound care. A 'silent epidemic' became a name for chronic wound incidence owing to its burden effects among nations; almost 2% of all populations in developed nations acquire chronic wounds in the course of their lives. The prevalence of chronic wounds and cardiovascular disease is very much alike, and the mortality rate of other wounds such as diabetic sour wounds is very comparable to that of cancer-related deaths. Because of reduced immunity, lower inflammatory mediators, and skin vulnerabilities, elderly persons are more likely to develop chronic wounds compared to younger ages [1]. Chronic wound statistics expect an increasing rate in the

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upcoming years, and this is because of numerous enhancing factors associated with age dependency ratio, obesity, persistent infection, and lack of target drug for diabetics, all of which create many social, clinical, and financial obstacles [2]. Indeed, the healthcare budget can be shortened in parallel with the increasing incidence rate of chronic wounds and their prevalence among nations. Recent data revealed that wound care costs exceed 4% of the health system budget. The United States is on top of the list with an estimated 126.8646 billion dollars in 2019, without including unreported wound costs managed by individuals [3].

Wound healing is a natural, intricate process involving biological, biochemical, and immune events that coordinate to restore the skin tissue damage and recover organ functionality. The healing process can be varied between individuals due to various interfering factors, such as poor immunity, any malnutrition, diabetic condition, any ischaemia, or ageing, which can compromise the healing process and cause difficulties for wound repair [4]. Wound recovery included mainly three stages: beginning with blood flow to injury site, followed by inflammatory cell infiltrations via increased capillary permeabilities (inflammation/granulation); reduced epithelialization and wound contraction (proliferation); and tissue repairing and strength regaining (remodelling). Growth factors such as TGFβ1 can play a crucial role in enhancing skin recovery, where they act as chemotactic molecules for attracting/repelling inflammatory molecules, cells, and fibroblasts near the injury site. Once released from Platelets and immune T cells after the skin penetration, TGFβ1 preserves the epidermal homeostasis via enhancing keratinocyte and macrophage aggregation, re-epithelialization, fibroblast infiltration, and directing endothelial and monocyte cells (participating in granulation phases), all together achieving faster wound recovery. Despite therapeutic innovation for wound management (such as intrasite gel), all have a similar molecular basis, and some of them are still under study because no specific treatment can cure all skin injuries as the general path. Legacy and topical therapeutics might not efficiently restore chronic wounds and their organ functionalities. Treatment options such as Negative pressure wound therapy, other bioengineered skin substitutes, and growth factor-based therapy are promising, but they are costly with reduced availability [5].

Medicinal plants and their numerous therapeutic uses in Iraq date back to 60 000 years, according to traces found in the Shanidar IV Neanderthal grave, where they apply flowers on their burials and practice natural medicine in the mountains of Iraq Kurdistan (an autonomous region). Since then, folkloric medicine has been a common therapy practiced by nomadic tribes living in Kurdish rural sites and villages, especially in the Sulaimania and Erbil provinces [6]. Moreover, the two cities are recognised by their large bazaars in downtown, where thousands of herbal therapeutic species are traded in more than one hundred herbalist shops and different natural therapeutic products consumed in various forms to date. Indeed, ethnobotanists declared that approximately 1500 plants were utilised as a therapeutic and aromatic source. Most of these species were from wild habitats, and a few were cultivated. Despite the popular use of herbal medicine in Iraqi Kurdistan, literature data on their phytochemical and biological potentials are scarce. In the last century, the drug discovery strategies run by the pharmaceutical industry mainly relied on synthetic organic chemistry, abandoning natural resources [7,8]. However, not achieving optimal efficiency and increasing effects, the last decades witnessed a resurrection of alternative approaches, plant-based therapeutics, and their new potential application [9]. Numerous plant-based biomolecules have been introduced as valuable commercial sources and used as nutraceuticals, cosmeceuticals, and pharmaceuticals. Moreover, modern drug-searching approaches include a continuous seeking for alternative natural sources, searching for underutilised herbal medicine, and exploring herbal therapies grown in under-looked geographical areas, such as those grown in developed countries with rich biodiversity [10,11]. Therefore, in recent decades, researchers extensively turned their focus on exploring new natural sources such as medicinal plants as a potentially viable source of drug formulation, and this study was a continuation of this search [12,13].

Anchusa is a well-known flowering plant that grows in sandy soil overlying saline flats and on the banks of rivers of the Mediterranean region [14], in which 9 species have been found in Iraq, especially in Diyala, Baquba, Liwa, and in Kurdistan, Haji Omaran district and the edge of shallow pools. These plants include *A. aegyptiaca, A. arvensis, A. italica, A. strigosa, A. hispida, A. italifolia, A. aucheri, A. officinalis, and A. limbata* [15,16]. *Anchusa officinalis* is a widely distributed flowering plant in the Mediterranean and Balkan countries. Previous ethnobotanical studies have indicated numerous traditional uses of *A. officinalis* as a therapeutic agent for astringent wounds, burns, bruises, and ulcers [17]. Moreover, ethnobotanists declared that all parts of *A. officinalis L.* have been used for therapeutic purposes, including sealing skin cuts, varicose veins, and stomach ailments [18]. The phytochemical profile (polyphenols, tannins, pyrrolizine alkaloids, and triterpenoids) has been linked with its major biological potentials, including antioxidant, antimicrobial, and anti-tumor actions [19], anti-inflammatory and antidiabetic actions [20]. Therefore, the current study evaluates the toxicity and wound-healing effects of APEAO on excisional-dorsal neck cuts in rats by different molecular assays.

2. Material and methods

2.1. Plant collection, identification, and extraction

The aerial parts of *A. officinalis* (Figure 1) were collected during spring 2020 from Erbil, Iraq (altitude: 36.30416, latitude: 44.42439). The plant was authenticated by Prof. Dr. Abdullah Sh. Sardar and voucher specimens (9030) were provided by Salahaddin University Herbarium-Education College (ESUH).

2.2. Extraction

Aerial parts of *A. officinalis* (100 g) were transferred into an Erlenmeyer flask of n-hexane (3×250 ml) with regular shaking in an ultrasonic bath (15 min), and then the mixture was left for 1 h at 25–37 °C. After filtration of the mixture using 0.45 *μm Millipore*

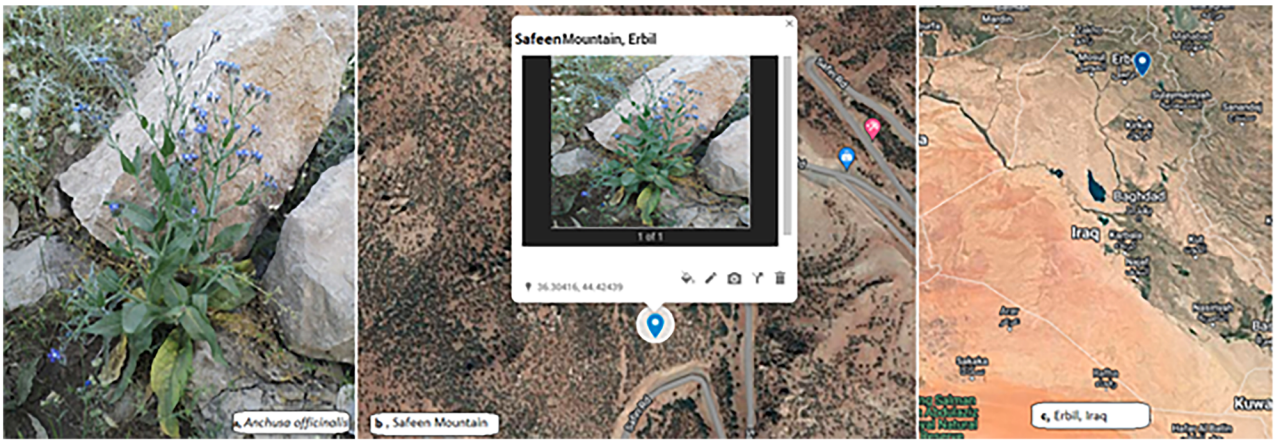

Figure 1. Aerial parts of *A. officinalis* (a) collected from Safeen Mountain (b), Erbil, Iraq (c).

filter paper, the solvent was separated using rotavapor (at ≤35 °C) at reduced pressure to afford a dried yield (11.9%) [21].

2.3. Chemicals

The chemical reagents, ELISA kits, and intrasite gel (Carboxymethyl cellulose, water, and propylene glycol) were purchased from Sigma Aldrich, China. Methanolic extracts of *A. officinalis* leaves were dissolved in 10% tween 20 and prepared in different concentrations as supplementary treatment.

2.4. Ethics approval

The animal care and use protocols were following the *ARRIVE* guidelines [22]. The experimental protocols were agreed upon by Tishk International University-Erbil/Ethics Committee (BIO.38, 23/12/2024).

2.5. Acute toxicity in experimental animals

The toxicity trial was conducted in compliance with OECD-423 guidelines [23]. In brief, Sprague Dawley rats (equal males and females), 7–8 weeks and weighted 180–200 g, were caged separately in 3 mesh-wired cages: group A rats ingested normal saline; group B rats received 2 g/kg of APEAO; group c rats supplemented with 5 g/kg of APEAO. Before treatment, all rats were fasted overnight, and after treatment, they were fasted again for an additional 3–4 h. The regular check-up began immediately after treatment and continued for 14 days for any possible incidence of toxic damage or physiological abnormalities (Mild tremors, convulsion, loss of righting reflex, impaired limb function, ataxia lethality, paresthaesia, choreoathetosis, rarely paralysis, frightened, eye colour change, biting, and shortness of breath) [24]. The physical characteristics (locomotion, eye colour, skin piloerection, excessive salvation, and exophthalmos) and daily water and food consumption were kept under sight throughout the procedure. Finally, rats received an overdose of 3 mg xylazine and 30 mg/kg ketamine and were sacrificed. Blood samples were biochemically analysed, and the liver and kidney organs were dissected for histopathological examination [25].

2.6. Experimental design of wound-healing study

2.6.1. Induction of wounds 'rats

Twenty-four male Sprague Dawley rats were randomly separated into four cages with free access to a standard diet and water. To create an excisional wound, all rats as small doses of intraperitoneal injection of 12.5 mg/kg xylazine, and 87.5 mg/kg ketamine were delivered to all rats [26]. After shaving and disinfecting their rat's dorsal neck, a small round cut (2.00 cm diameter) was formed using a round sharp seal (Figure 2) [27]. For the following two weeks, the rat's wound was addressed with 0.2 ml of the following (2x daily treatment):

Group A rats had 10% tween 20.

Group B rats were addressed with intrasite gel.

Group C and D rats received 250 and 500 mg/kg of APEAO.

The wound size was evaluated at day 0, 5th, 10th, and 15th after excision using partial anaesthetisation, a highlighter, and a square paper (1 mm^2). The closure % was determined using the equation below:

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

After a two-week trial, all rats received an overdose of intraperitoneal anaesthesia injection of 30 mg/kg xylazine and 3 mg/kg ketamine and were sacrificed by cervical dislocation.

The skin tissues from the healed area were excised for histopathological evaluation, and the blood was collected by intracardiac puncture for analysing separated serum samples for their inflammatory cytokines [28].

2.6.2. Histology of wound tissues

The regenerated tissues from the healed area were analysed for histopathologic alterations by applying a previously detailed procedure. In brief, small tissue pieces were soaked in phosphate (10%)-buffered formalin before transferring into an automated tissue processing machine, including several processes of paraffinization, tissue fixatives, washing with distilled water, dehydration, clearing, and infiltration. After the skin tissue preparation, they were deepened in haematoxylin and eosin. The stained slides were incubated overnight and

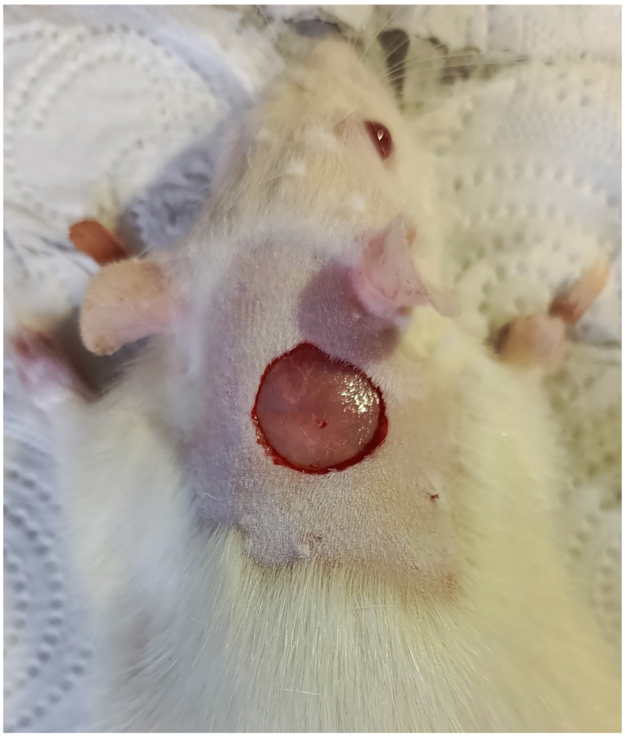

Figure 2. The excisional wound was created on each rat's dorsal neck at day zero.

screened under a Nikon light microscope (at 2× and 100×) to elucidate any structural tissue changes, including collagen formation, inflammatory cell infiltration, fibroblast proliferation, epithelialization, and neovascularization [28].

2.6.3. Immunohistochemistry

The obtained skin samples were soaked in 10% formalin and then transferred into an automated tissue homogeniser at 4 °C for 10 min (Polytron, Germany) [29]. The tissue homogenate mixture was examined for TGF-β1 contents (ng/ml) using commercially available Elisa kits for TGF-β1 (E-EL-0162, Biotech Co., Ltd, Wuhan).

2.6.4. Enzyme activity in skin homogenate

The skin tissue sections from the healed area were homogenised at 4 °C for 10 min using a Teflon homogeniser (Polytron, Germany). The supernatant was transferred into separate tubes and analysed for the antioxidant enzymes contents (CAT, C0979; SOD, SAB4200807; GPx, AV41491) and non-enzymatic contents (MDA, SAB5202544) contents according to the standard procedure available on ELISA kits [28]. To estimate the hydroxyproline content in recovered skin tissues, skin pieces were homogenised (4 °C for 10 min) and centrifuged for 5 min at 5000 rpm. After separating the supernatant, they were analysed for the HXP content using a commercial ELISA kit (Sigma Aldrich, China), applying protocols found in the kit's catalogue, including specificity, standardisation, and precision [30]. The skin tissue homogenates were also examined for the HXP contents using an available ELISA kit (MAK008) and the company's standard protocols (Sigma Aldrich, Germany).

2.6.5. Inflammatory cytokine

The intracardiac blood samples were centrifuged at 5000 rpm for 10 min. The inflammatory cytokines, including Rat TNF-α ELISA Kit (RAB0480-1KT), IL-6 ELISA Kit (RAB0311), and IL-10 (RAB0246) were estimated in the separated supernatant using commercially available kits for the ELISA machine (Sigma Aldrich, China). The complete protocol followed the sandwich methods of ELISA, provided by the Producer's company [31].

2.7. Statistical analysis

The laboratory data analysis was conducted using one-way ANOVA, SPSS, and Duncan's multiple ranges between groups. The figures were designed using Graph Pad Prism 9.0. The results were expressed as mean ± SEM, and the significance level was set at $p < 0.05$.

3. Results

3.1. Acute toxicity

The present safety consumption of APEAO demonstrated non-toxic effects in rats supplemented at doses 2 and 5 g/kg. Supplemented rats showed normal behaviour without experiencing any morbidity or mortality during or after the trial. The physical changes, including alteration of skin and eye colour, convulsion, lethargy, exophthalmos, and physiological alteration, were absent in APEAO-treated rats compared to normal control rats. The water and food consumption, as well as the body weight of supplemented rats, were very comparable to that of normal controls. The histopathological evaluation of obtained liver and kidney tissues showed similar tissue layer arrangements between APEAO-ingested and normal saline-treated rats (Figure 3). The serum profiling of APEAO-treated rats revealed normal amounts of biochemical contents, found within normal range and comparable to normal control rats (Available on request). The above results indicate the non-toxic effect of APEAO up to 5 g/kg, providing scientific evidence to explore the plant species for different biological potentials in different *in vivo* trials.

3.2. Wound experiment

3.2.1. Proportion of wound contraction

The results showed different levels of wound closure in experimental rats as a result of different treatment strategies based on wound evaluations on the 5th, 10th, and 15th day of excisional neck injury (Table 1). In the present wound trial, all rats showed noticeable wound size reduction and skin recovery, except for the vehicle rats, which exhibited the lowest wound contraction throughout the procedure. The vehicle rats, as expected, exhibited the highest opened skin area and lowest closure proportion (16.03, 59.54, and 67.33%, respectively) during all three timed evaluations compared to other treated rats. In contrast, rats treated with APEAO (0.2 ml of 250 and 500 mg/kg) revealed a gradual reduction in wound size and increased wound closure percentages in a dose-related manner compared to that of positive controls. Moreover, 500 mg/

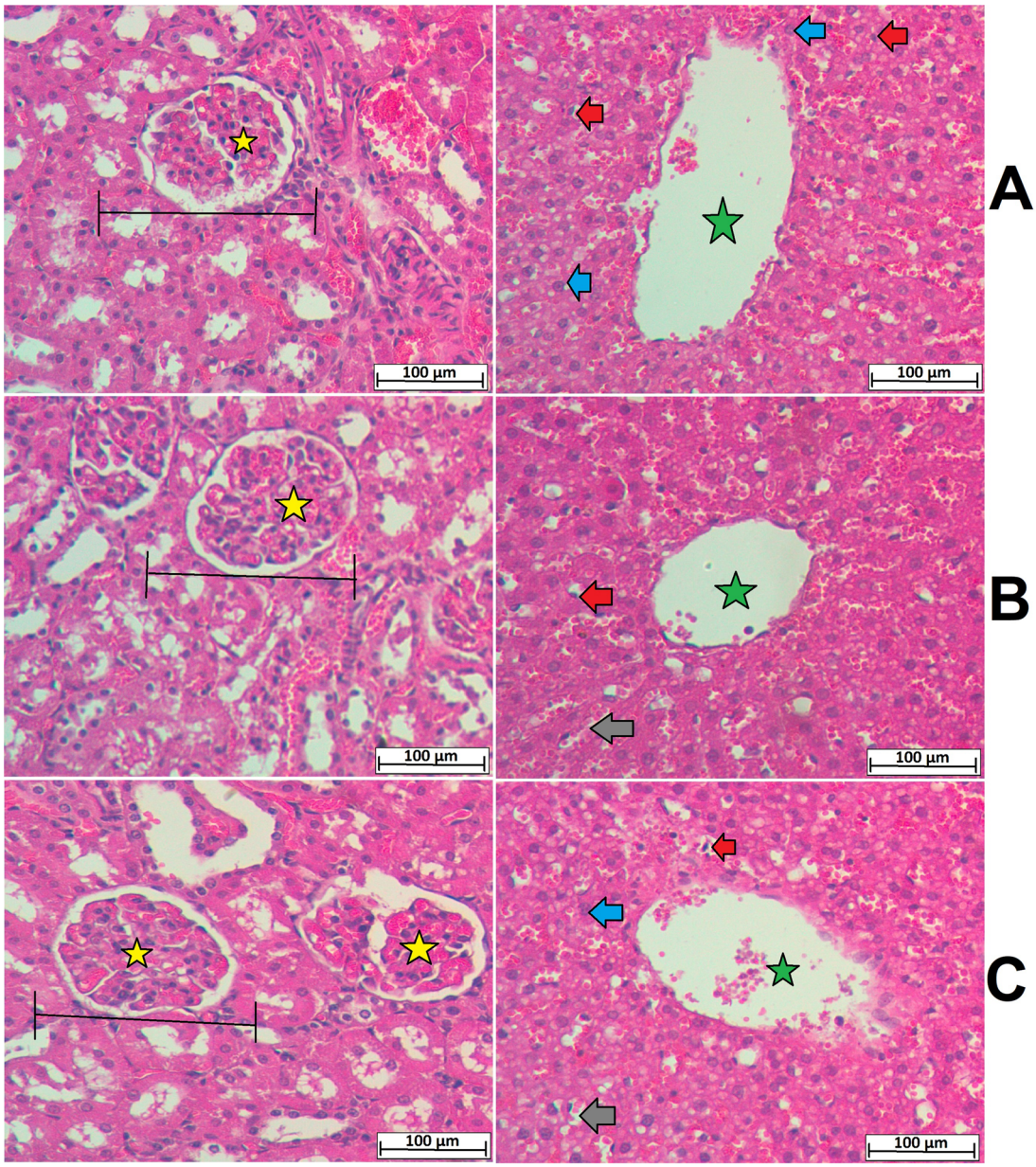

Figure 3. Microscopic views of liver and kidney of APEAO and normal saline-treated rats. Group A rats had normal saline; group B rats were supplemented with 2 g/kg of APEAO; group C rats had 5 g/kg of APEAO. Red arrow, Kupffer cell; blue arrow, round-nucleated hepatocyte; gray arrow, sinusoids; green star, central vein; black line with double head, bowmen's capsule with glomerulus; yellow star, glomerulus. The structural tissue arrangement of liver and kidney organs was comparable between experimental rats (Haematoxylin and eosin stain, 20x).

kg APEAO-treated rats exhibited significant therapeutic potentials and maximum and fastest skin contraction (92.71%), particularly after the 15th day of excision, significantly varied compared to positive controls (67.33%) and very comparable to that (94.13%) of intrasite gel-treated rats. The wound estimation results indicate significant therapeutic potentials of APEAO in accelerating wound closure during the mid and final stages of excisional cuts.

The gross morphology evaluation showed decreased wound contraction areas in 10% tween 20-treated rats throughout the experimental period, which was significantly varied from that of intrasite gel or APEAO-treated rats (Figure 4). Dorsal neck wounds addressed with 0.2 ml of intrasite gel or APEAO revealed accelerated wound healing actions during all three periodic times of wound estimations, showing a gradual effect as the procedure continued. Wound estimations on days 10 and 15 were comparable between APEAO (0.2 ml of 500 mg/kg) and intrasite gel-treated rats. The proportion of wound contraction (92.36%) in APEAO (0.2 ml of 500 mg/kg)-treated rats was statistically higher than that (66.89%) of 10% tween 20-treated rats, indicating the role of this plant extract in improving wound healing process and skin tissue regenerations (Table 1, Figure 4).

3.2.2. Effects of APEAO on tissue structure

The skin tissue sections (H&E-stained) obtained from healed areas of different experimental rats were screened using different magnifications (Figure 5). Microscopic skin tissue examination showed different levels of tissue granulation and fibroblast proliferation as a result of different wound management protocols. The vehicle rats were marked with the lowest tissue granulation, elevated inflammatory cell reposition, reduced

Table 1. Effect of APEAO on wound size and closure proportion in excisional rat model.

Clusters	Wound area mm² Day 0	Wound area mm² Day 5	Day 5 Closure %	Wound area mm² Day 10	Day 10 Closure %	Wound area mm² Day 15	Day 15 Closure %
A	225 ± 0.32	188.93 ± 3.2^{c}	16.03^{c}	91.02 ± 2.8^{a}	59.54^{c}	73.50 ± 2.6^{c}	67.33^{b}
B	225 ± 0.20	82.34 ± 2.4^{a}	63.40^{a}	32.40 ± 2.4^{a}	85.60^{a}	13.19 ± 2.5^{a}	94.13^{a}
C	225 ± 0.21	105.43 ± 3.5^{b}	53.14^{b}	53.10 ± 2.5^{b}	76.4^{b}	23.24 ± 2.9^{b}	89.67^{a}
D	225 ± 0.32	98.55 ± 3.5^{b}	56.20^{b}	39.21 ± 3.4^{a}	82.70^{a}	16.39 ± 2.4^{a}	92.71^{a}

Group A was treated topically with 10% tween 20; group B was treated with 0.2 ml of intrasite gel; C and D, rats were treated with 0.2 ml of 250 and 500 mg/kg of APEAO. The same Letters on numbers within the column mean non-significant at $P < 0.05$.

epithelialization, and edoema, ultimately delaying wound-healing action. In contrast, topical application of intrasite gel or APEAO (0.2 ml of 250 and 500 mg/kg) revealed significant tissue recovery and inflammation termination represented by marked fibroblast proliferation, increased capillaries and epithelization, elevated vascularisation, and less inflammatory reposition compared to vehicle rats. Moreover, collagen deposition and dermal tissue growth were significantly varied between experimental groups. Vehicle rats revealed the lowest collagen deposition and tissue immaturity, indicating delayed early stages of wound repair. While intrasite gel or APEAO topical treatment accelerated the wound-repairing process by enhancing more collagen deposition, fibroblast attraction, and formation of collagens near the wound site, ultimately accelerating wound contraction. Moreover, APEAO addressing at 500 mg/kg accelerated structure tissue recovery by enhancing fibroblast dispersion in a more ordered pattern in granulated tissue, similar to intrasite gel treatment and significantly varied compared to that of positive control (Figure 5 and Figure 6).

3.2.3. Effect of APEAO on TGF-β1 expression in skin tissues

The histopathological analysis of recovered skin tissues obtained from different treated rats showed an extended range of TGF- β1 cytokine content, indicating different levels of tissue regeneration, tissue remodelling, and autoimmune responses. Vehicle rats showed the lowest TGF- β1 content (17.3 ng/ml) in their wound tissues, denoting delayed cellular processes such as inflammation, angiogenesis, fibroblast proliferation, collagen formation, and modulation of the extracellular matrix. All of which are required for achieving faster wound recovery. In contrast, rats managed with intrasite gel or APEAO exhibited increased TGF-β1 concentrations (43.5, 26.4, and 49.78 ng/ml) in recovered skin tissues that provoked the mentioned cellular processes needed for faster wound contraction (Figure 7).

3.2.4. APEAO effects on oxidative stress in skin tissues

The experimental rats showed different levels of oxidative stress as a result of various topical treatments in excisional rats (Figure 8). Vehicle rats experienced oxidative stress, indicated by reduced SOD (4.32 U/mg), GPx (5.21 um ml/min/mg), and CAT (26.14 um ml/min/mg) enzymes and elevated MDA (9.88 nmol/mg) contents in tissue homogenates. In contrast, intrasite gel or APEAO (0.2 ml of 250 and 500 mg/kg) topical treatment lowered oxidative stress and ROS generation, denoted by elevated SOD (15.40, 8.71, 10.25 U/mg, respectively), GPx (17.83, 7.9, and 14.5 um ml/min/mg, respectively) and CAT (52.43, 39.71, 45.83 um ml/min/mg, respectively) levels compared to that of vehicle rats.

The lipid peroxidation rate was significantly higher in vehicle rats, represented by higher MDA (9.88 nmol/mg) contents in their skin tissue homogenates compared to all treated rats. Rats addressed with intrasite gel or APEAO experienced less lipid peroxidation damage marked by less MDA content (2.70, 5.64, 3.21 nmol/mg) compared to the vehicle rats. The above results indicate marked antioxidant potentials of APEAO that could be linked with its accelerated potentials on the wound healing action (Figure 8).

3.2.5. APEAO effect on hydroxyproline

The wound tissue homogenates showed different levels of hydroxyproline (major amino acid of collagen) contents as a result of different topical treatments (Figure 9). The hydroxyproline content in skin tissues of vehicle rats (48.35 mg/g), indicated reduced collagen formation in granulated tissues that negatively impacted the wound healing process. In contrast, intrasite gel or APEAO (0.2 ml of 250 and 500 mg/kg) addressed rats exhibited increased hydroxyproline (86.49, 55.21, and 77.43 mg/g, respectively) in their skin homogenates as markers of increased collagen formation and cellular proliferation. The results indicate significant stimulatory potentials of APEAO on HXP generation, an important component of the extracellular matrix essential for faster wound contraction (Figure 9).

3.2.6. Effect of APEAO on inflammatory cytokines

The serum inflammatory chemicals are found to be significantly different between excisional wounded rats as a result of different treatment strategies. The vehicle rats showed increased TNF-α (167.24 pg/ml) and IL-6 (59.32 pg/ml) and decreased IL-10 (283.40 pg/ml) contents. In contrast, APEAO treatment (0.2 ml of 250 and 500 ml/kg) caused negative modulation of pro-inflammatory chemicals TNF-α (95.32 and 62.89 pg/ml) and IL-6 (43.75 and 22.41 pg/ml) and up-regulation of IL-10 levels (482.13 and 624.81 pg/ml), which was significantly varied compared to normal control rats (Figure 10). As expected, rats addressed with intrasite gel (group B) experienced the lowest tissue inflammation indicated by improvement in the serum inflammatory mediators (TNF-α, 46.98; IL-6, 14.53; IL-10, 831.45 pg/ml). The outcomes present APEAO as an effective anti-inflammatory agent that could be linked with its accelerative potential on skin wound healing.

4. Discussion

Natural products can have numerous therapeutic potentials, however, some of them can have poisonous effects, especially if they are consumed in large quantities or taken in

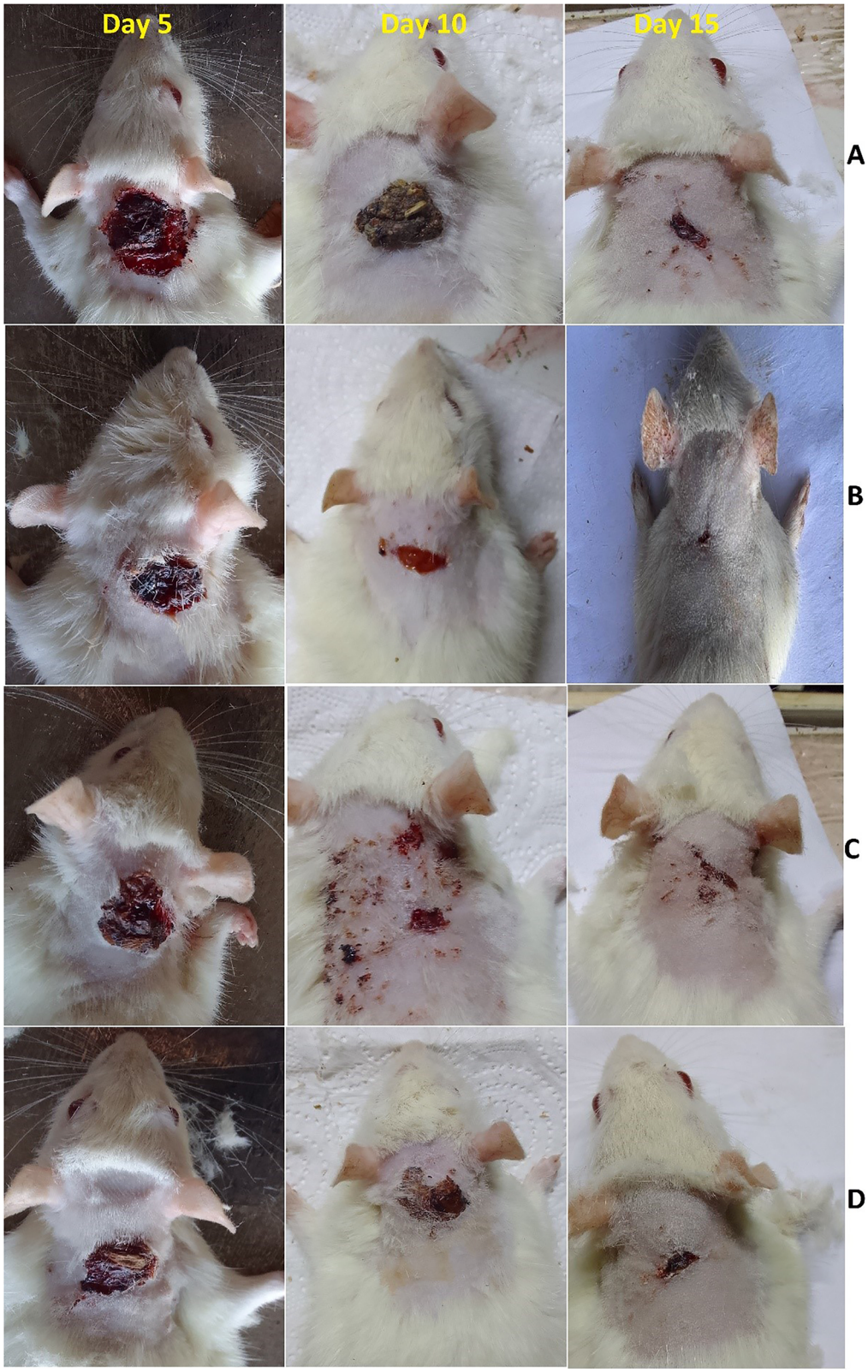

Figure 4. Effect of APEAO on wound closure photographed on days 5th, 10th, and 15th after excision. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). Vehicle rats showed larger wound sizes (mm2) and reduced wound proportion closer to treated rats. Intrasite gel or APEAO accelerated wound recovery and tissue regeneration, as indicated by smaller open wound areas and increased wound contraction percentiles.

repetitive doses. Therefore, toxicological evaluation is considered a first laboratory safety check for any plant of therapeutic interest [32]. The present toxicity estimation of *A. officinalis* indicated the safe consumption of APEAO (2 and 5 g/kg) in animal models based on the comparable histological and biochemical characteristics between normal control and APEAO-ingested rats. Similarly, previous scientists revealed non-toxic effects of *Anchusa* species (250 and 500 mg/kg

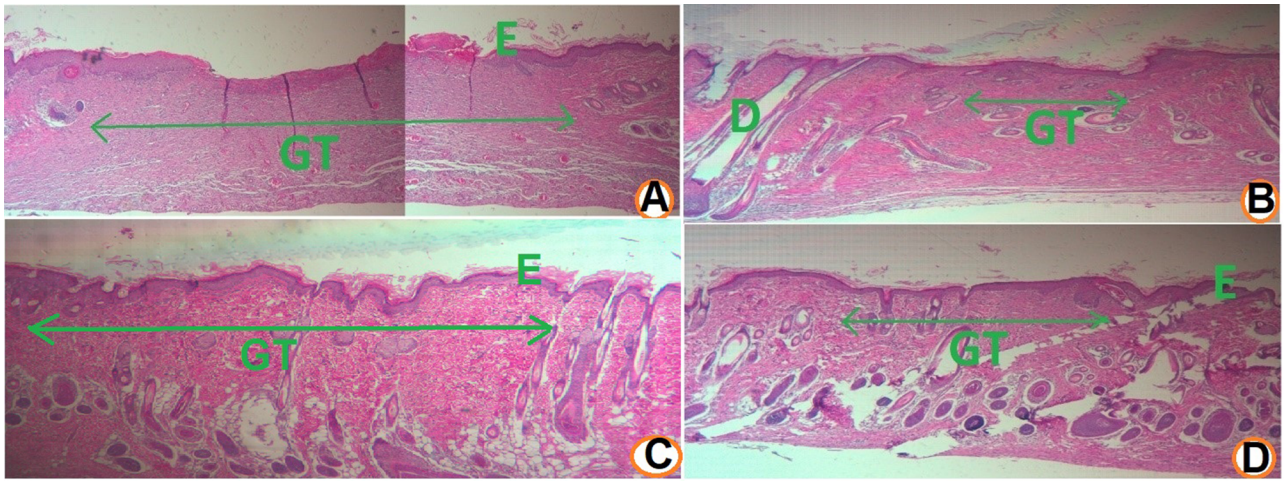

Figure 5. Microscopic views (4x) of Skin tissue layers of rats on day 15th after excision. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). The vehicle rats showed the largest open wound area (green line), reduced skin tissue regeneration, increased inflammatory cells, and reduced tissue granulation. Intrasite gel or APEAO topical treatment enhanced wound closure by attracting more fibroblasts to granulated areas, facilitating inflammatory phase termination and tissue maturation. E, epidermis; GT, granulation tissue; D, dermis.

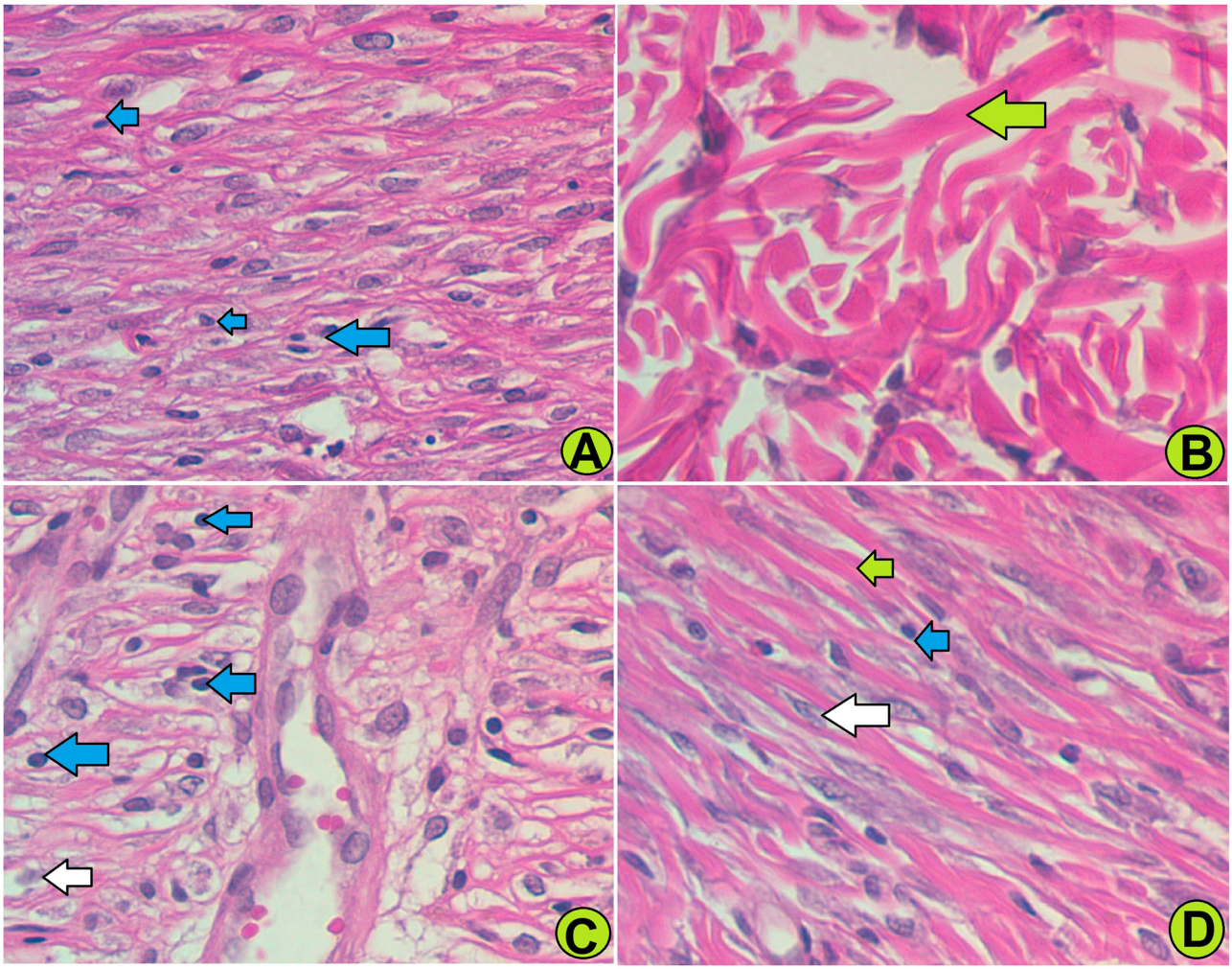

Figure 6. Microscopic views (40x) of Skin tissue layers of rats on day 15th after excision. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). The vehicle rats showed increased inflammatory cell infiltration, lowest fibroblasts, and collagen deposition. Intrasite gel or APEAO-treated rats exhibited more fibroblast regeneration in granulated areas and more collagen formation that aided in inflammatory phase termination and tissue maturation.

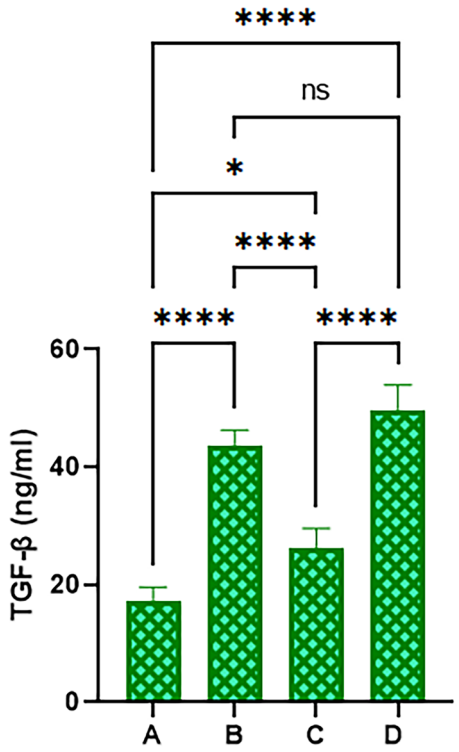

Figure 7. Effect of APEAO on the TGF- β1 contents in skin tissues on day 15th after excision. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). Vehicle rats showed the lowest TGF- β1 cytokine expression in their skin tissues, indicating early phases of wound repair when many cellular processes are yet to be activated. Intrasite gel or APEAO caused up-regulation of TGF- β1 in recovered tissues, which ultimately enhanced many cellular processes required for achieving faster wound repair.

extracts) in rats, in which rats were completely functioning with zero mortality cases even after 30 days of toxic trial [33]. Accordingly, rats supplemented with crude extracts from *Anchusa azura* 200, 400, 600, 800, and 1000 mg/kg did not show any indications of toxic incidence based on biochemical and histological evaluations [13]. According to the OCED and Hodge and Sterner measurements, the above data could be enough to support the safe utilisation of *Anchusa officinalis* extracts, Therefore, we have attempted to evaluate its therapeutic potential as a wound healer in a two-week daily treatment trial.

Injury recovery is a multistage plus self-motivated scheme of regaining cellular organisation and tissue regeneration intimately as likely as its ordinary state. Wound contraction occurs during the remedial course, commencing in the fibroblastic stage where injury vicinity starts to decrease. The proliferation phase is characterised by angiogenesis, collagen deposition, tissue granulation, epithelialization, and gash-tightening, resulting in a smaller amount of observable scar tissue. In the present wound trial, the results elucidated that APEAO-treated injury exhibited a noticeable hastening acceleration of wound recovery and a remarkable reduction of lesion vicinity at days 10th and 15th after surgery contrast with positive control indulgence injury. Moreover, rats addressed with APEAO did not show any sign of ache, frustration, restiveness, biting, or scraping of the injury site. The histological evaluation (using H&E) of recovered skin tissues from APEAO-treated rats showed increased epithelialized skin tissues, neovascularization, fewer inflammatory cells, increased granulations including fibroblasts, and more organised collagen release compared to vehicle indulgence rats. Addressing wounds with APEAO enhanced the initiation of a clear coverage on open wound areas that thickened skin layers (epidermis) and prevented further tissue damage, which could be linked with its phytochemical profiles (polyphenols, tannins, pyrrolizine alkaloids, and triterpenoids) as shown previously 19. Similarly, investigators have shown a complete healing action of *Anchusa* species as a crude extract and as a combination therapy (quercetin 3-O-rutinoside and ellagic acid) at a concentration of 50 µg/mL, which were linked with pyrrolizidine abundance in Anchusa species [34]. Accordingly, researchers have shown the wound-healing potentials of different pyrolizidine and tannins in the shutdown of the inflammatory response and acceleration of wound contraction in different animal trials [35,36].

The wound-healing process requires several growth factors for chemotactic actions needed for the aggregation of inflammatory and fibroblasts near the injury site (tissue granulation). TGFβ1 is a well-known growth factor released by Platelets and immune T cells once the skin is penetrated that maintains epidermal homeostasis and is involved in wound contraction through enhancing keratinocyte and macrophage attraction, fibroblast infiltration, re-epithelialization, and directing endothelial and monocyte cells [37]. Moreover, the TGF β1 protein is essential for activating α-smooth muscle actin to initiate myofibroblast formation, thereby contracting collagen fibres and accelerating wound closure. Meanwhile, another link of TGF β1 with wound healing acceleration has been anticipated through its stimulatory potential on angiogenesis by attracting growth factors to the wound area [38]. The provoking action of TGF β1 on the re-epithelialization has been explained through its enhancing role of keratinocyte migration after the binding of ligands on cell surface receptors. In light of the above knowledge, the present results elucidated the provoking efficacy of APEAO on the TGF-β1 expression by the keratinocytes in skin tissues, which could be correlated with its chemical constituents (mainly pyrrolizidine, phenolics, and tannins) as previously explained [39]. Accordingly, researchers have shown significant up-regulation of TGF-β1 provoked by pyrrolizidine alkaloids in a four-month rat trial [40].

Reactive oxygen species (ROS) are valuable enhancer factors required for the initiation of many cellular processes during the early stages of wound healing. Indeed, to an extent, oxidative stress and ROS formation are indispensable factors for faster wound recovery, and their total suppression can halt the overall cellular processes. Scientists declared that limited hydrogen peroxide formation can have a provoking impact on the healing process, and its total inhibition (using synthetic catalase) has negatively affected the vascular endothelial growth factor generation and tissue regeneration, thereby impairing the wound recovery. Despite being a valuable mediator of many cellular mechanisms and inflammatory processes, increased ROS generation can have undesirable consequences, such as skin ischaemia (a skin disorder characterised by reduced blood supply, enhancing more ROS generation and leukocyte

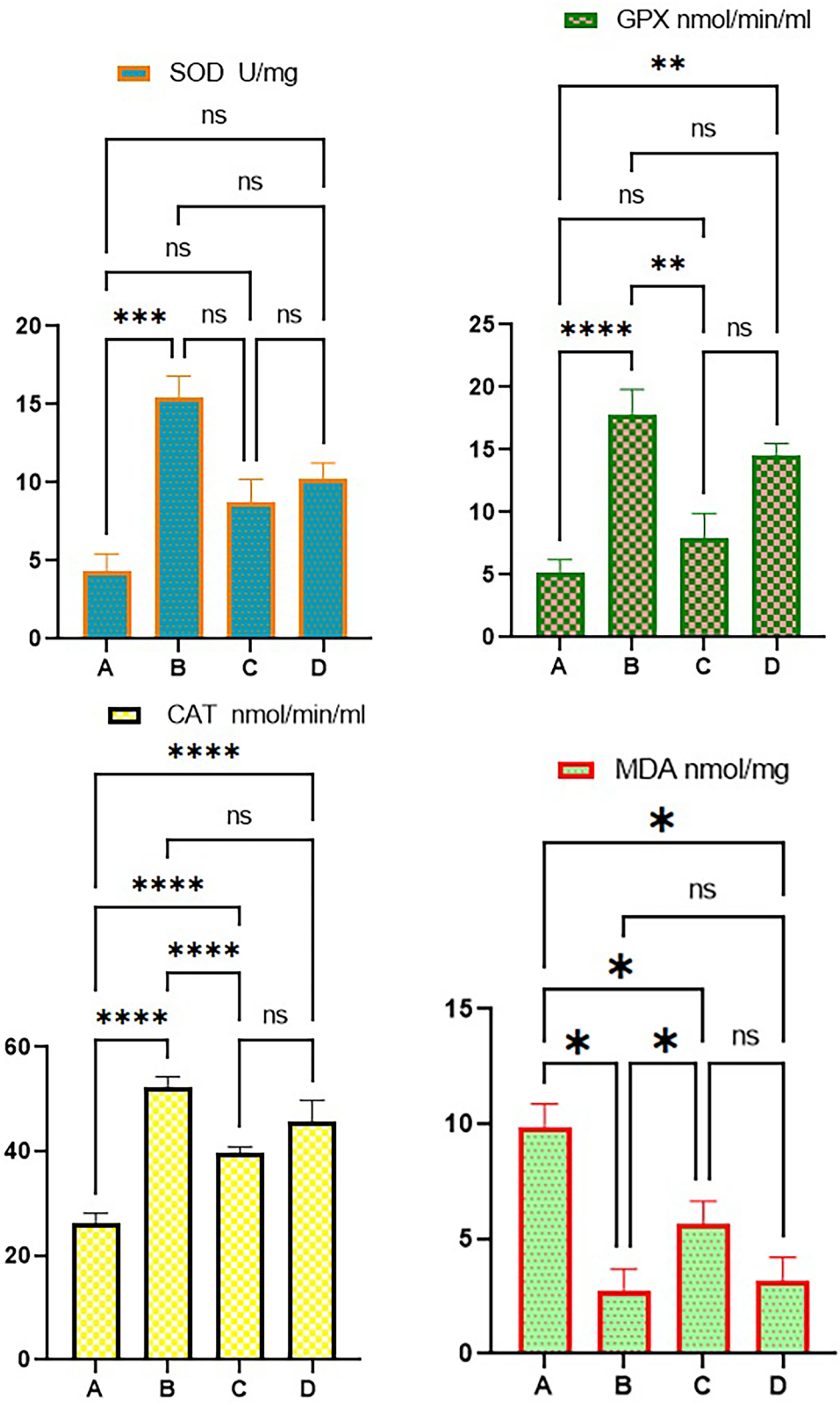

Figure 8. Effect of APEAO on oxidative stress in excisional wound rats. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). The asterisk on the bars mean significance at $p < 0.05$.

multiplication near wound beds that result in delayed wound recovery because of elevated inflammation and oxidative stress [41]. To overcome this, the body generates endogenous antioxidants, which in some cases, as chronic wounds, cannot keep up with the increased release of free radicals, making skin more prone to structural and functional alterations. As an endogenous antioxidant enzyme, SOD help in the reduction of ROS formation by the dismutation of O^{2-} to H_2O_2 and O2.

Similarly, the catalase enzyme converts H_2O_2 to H_2O and oxygen with the help of cofactor iron or manganese, reducing the odds of oxidative stress incidence [42]. The present data analysis revealed significant antioxidant potentials of APEAO shown by elevated SOD and CAT levels in repaired wound tissues, which may facilitate faster wound contraction in APEAO-addressed rats. Similarly, researchers have shown significant total antioxidant activity (90.26 ± 0.99 µg AA/g) and lipid peroxidation suppression (35.45 ± 1.34 IC50 µg/ml) of ethanolic extract of APEAO, which were correlated with chemical contents of APEAO, namely phenolics (104.03 ± 0.63), flavonoids (30.26 ± 0.40), and tannins (Ethanol 70.67 ± 0.51). Moreover, *A. officinalis* nano-filtrate exhibited significant scavenging activity (IC_{50} = 0.0032 mg/mL), very similar to that (IC_{50} = 0.0036 mg/mL) of reference ascorbic acid. These bioactivities of APEAO were correlated with its polyphenolic profile (Chlorogenic acid, rosmarinic acid, and luteolin) [20]. Accordingly, increased wound healing potentials of *A. azurea* extracts have been linked with the antioxidant properties of its phytoconstituents, namely phenolic, flavonoid, fatty acids, and terpenoids [13].

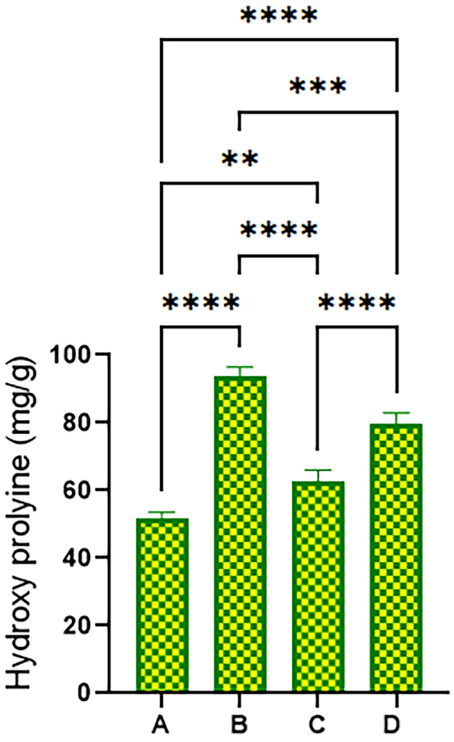

Figure 9. Effect of APEAO on the hydroxyproline content in wound tissues. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). Vehicle rats showed fewer HXP content in their wound tissues, indicating reduced collagen content. In contrast, APEAO topical treatment enhanced HXP release in skin tissues, ultimately resulting in higher collagen formation essential for faster wound recovery.

MDA is a well-known reactive compound formed by polyunsaturated fatty acids oxidation, which is relayed as a biomarker of lipid peroxidation [43]. The present study revealed reduced MDA content in the recovered skin of APEAO-treated rats, denoting the antioxidant potential of plant chemicals (polyphenols, tannins, pyrrolizine alkaloids, and triterpenoids) that aid in a faster wound-repairing process. Accordingly, numerous previous studies showed significant inhibitory potentials of *Anchusa* chemicals in the negative modulation of lipid peroxidation biomarkers in a variety of laboratory trials [44–46]. Similarly, the ethanol extract of *Anchusa officinalis* showed significant lipid peroxidation reduction (IC_{50}: 35.45 ± 1.34 µg/ml), which is more significant than extracts from non-polar solvent (chloroform) [47].

The wound-healing process is characterised by an increased generation of collagen protein, an important component of

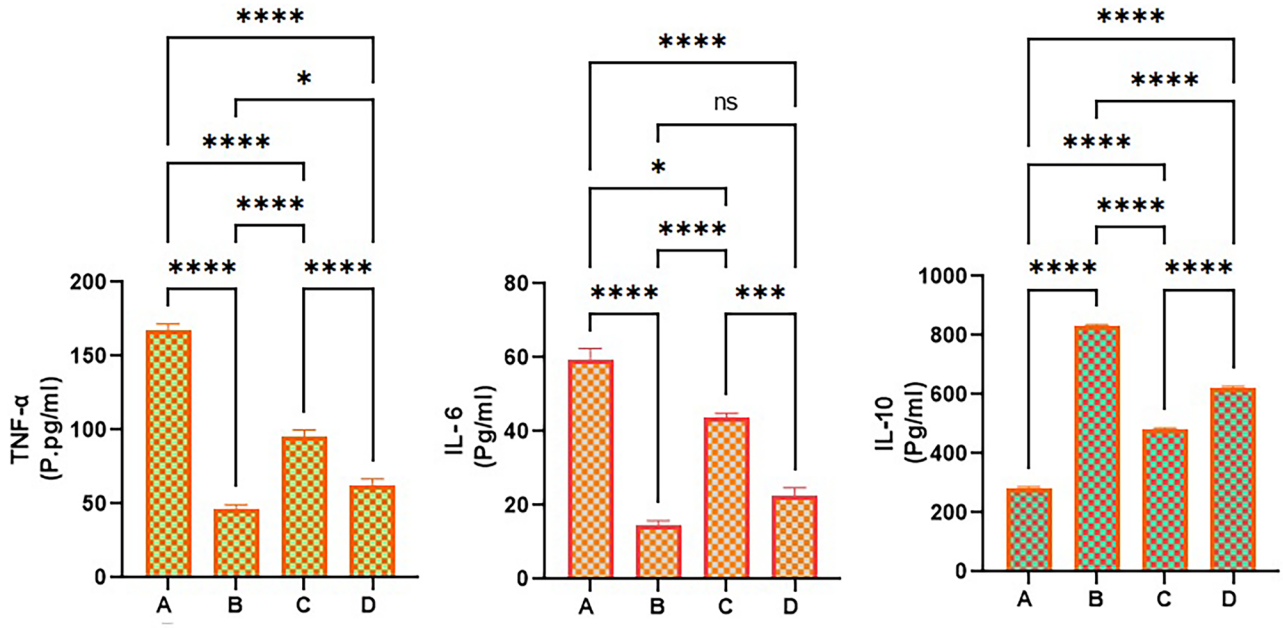

Figure 10. Effect of APEAO on the serum inflammatory indicatory in excisional-injured rats. Group A rats were treated with 10% tween 20; group B rats addressed with 0.2 ml of intrasite gel; group C rats managed with 0.2 ml of low dose APEAO (250 mg/kg); group D rats received 0.2 ml of high dose APEAO (500 mg/kg). The vehicle rats exhibited the highest inflammatory condition, ultimately negatively affected the wound-healing process. APEAO topical treatment revealed noticeable anti-inflammatory potentials represented by negative regulation of serum TNF-α and IL-6 cytokine and positive modulation of IL-10 chemicals, thereby terminating the inflammatory phase of wound healing and ultimately enhancing a faster wound process.

growing cells. As a major amino acid constituent (13.5%) of collagen, HXP can preserves the twisting structure of collagen, becoming more stable and resistant to stress factors. HXP is a valuable marker of college concentration in regenerated skin tissues essential for the final two phases of wound closure, enhancing faster wound restoration. Moreover, increased collagen content not only provides strength and integrity to the skin matrix but is also a valuable co-factor for cellular homeostasis and epithelialization during the progressing stage of wound healing. The present wound-healing was significantly provoked by topical treatment of APEAO because all the parameters related to healing were significantly modulated including up-regulation collagen formation. Similarly, researchers have shown significant potentials of pyrrolizidine-rich plants in positive regulation of HXP concentrations in a hepatic obstruction-induced rat model, indicating the role of this immunohistochemical along with TGF-β levels and anti-inflammatory cytokines in the blockade of the inflammatory-related diseases [48]. Previous data revealed that plant phytochemicals, namely phenolics, flavonoids, and tannins, are effective positive modulators of hydroxyproline in different animal trials, which were linked with faster wound contraction [49–51].

At the injury site, the localised formation of inflammatory cytokines stimulates the wound-repairing process through the facilitation of monocyte and T-lymphocyte aggregation (chemotaxis). During the initial stages, inflammatory cytokines (IL-1β, IL-6, and TNF-α) are generated mainly by active macrophages, indicating an important role of these defensive cells in progressing wound recovery [32]. At the later phase of wound recovery, inflammatory cytokines are further released by leukocytes, platelets, and macrophages. Despite their important role in the early wound phases, an increased pro-inflammatory mediator can extend the inflammatory stage, thereby delaying wound recovery. Consequently, chronic wounds may initiate as a result of proteinase enzymes denaturing these chemical mediators. IL-10 cytokine is ubiquitously secreted by numerous cells, including lymphocytes, granulocytes, and keratinocytes, that suppress Th1 cytokines' action and co-activate macrophages. Moreover, IL-10 can block the NF-κB pathway and can regulate many cellular processes such as JAK-STAT signalling, inflammation, and autoimmune pathways, lowering hemodynamic alteration and inhibiting pro-inflammatory cytokines, resulting in less aggregation of inflammatory cells near the injury site [43]. Moreover, mucosal layers can generate more IL-10 and TNF-α during skin tissue regeneration, and once the IL-10 levels are enough, it can inhibit TNF-α action so that the next healing stage began (proliferation) [52]. In the present trial, In the present work, topical application of APEAO on excisional injured rats caused significant immunomodulatory actions shown by lower TNF-α and IL-6 levels and higher IL-10 chemicals compared to vehicle I rats, which could be due to *Anchusa* metabolic (Pyrralizidine) potentials in regulating molecular mechanisms associated faster skin regeneration. Accordingly, researchers have reported a noticeable anti-inflammatory action of *Anchusa officinalis* in an in-vitro trial, which is mainly linked with its increased polyphenolics (luteolin, chlorogenic acid, rosmarinic acid, rutin, and isoquercitrin) [20]. Similarly, 200 and 400 mg/kg of *Anchusa* extracts ameliorated inflammation in Carrageenan-mediated paw edoema in animal models, which was mainly linked with its polyphenolic contents (phenolic, gallic, caffeic, and chlorogenic acids) as a pharmacologic agent [53]. The present trial limitations included facility shortage, availability of essential kits and equipment, and small budget. Therefore, further investigations should be conducted on these potential wound healers to unveil their exact mechanisms and isolate the main compounds responsible for their biopotentials. Furthermore, applying plant extracts as a therapeutic wound healer faces challenges and difficulties, such as reduced solubility and bioavailability and the absence of broad clinical trials on the long-term applications.

5. Conclusion

In this study, we evaluated the acute toxicity and wound-healing potentials of APEAO in animal models. The histological and biochemical indicators showed non-toxic effects of APEAO in rats ingested with up to 5 g/kg. In a two-week wound trial, the results suggest significant wound healing potentials of APEAO mediated by its modulatory actions on oxidative stress (increasing SOD, CAT, and GPx and reducing MDA) and inflammatory (IL-6, TNF-α, and IL-10) mediators consequently leading to increased re-epithelization, fibroblast proliferation, and collagen deposition (HXP). Moreover, APEAO-treated rats exhibited increased TGFβ1 protein in their skin tissues, resulting in more organised granulated tissues and cellular actions that aided in faster wound recovery. These outcomes encourage pharmaceutical companies to reconsider remedies formulated with these natural sources and contribute more to the environmentally sustainable commercial investigation of these natural sources.

Author contributions

M.A.A, A. A.j., conceptualisation; A.A.J., investigation; T.S.A., M.I.S., P.A.I., S.M.A., M.H.M.R., formal analysis and software; R.R.H., S.M.A., B.A.W., resources and validation; A.A.J., P.A.I., H.I.A., design and review; A.A.J., writing manuscript; all authors agreed on final version of the manuscript.

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Data availability statement

Further details will be available on request.

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