



Wound healing potential of *Canarium luzonicum* in rats: Role of caspase-3 modulation

Yahya I. Asiri^{ID}, Krishnaraju Venkatesan^{ID}

Department of Pharmacology, College of Pharmacy, King Khalid University, Abha, Asir, Saudi Arabia

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ABSTRACT

Introduction: The process of wound healing is intricate and includes tissue remodelling, cell proliferation, and inflammation. Elemi essential oil (EEO) from *Canarium luzonicum* exhibits anti-inflammatory, antibacterial, and antioxidant properties, yet its wound-healing efficacy in vivo remains untested. This study aimed to evaluate the therapeutic potential of EEO in promoting wound healing in rats.

Methods: Male Wistar rats were assigned to EEO-treated (10% w/w), reference-treated (1% silver sulfadiazine), and control (soft paraffin) groups (n=6 each). Full-thickness dorsal wounds (2 cm) were created. Wound contraction was monitored on days 1, 4, 8, 12, 16, and 21. Serum interleukin-1-beta (IL-1 β), tumour necrosis factor-alpha (TNF- α), and CD68 were measured via enzyme-linked immunosorbent assay (ELISA). Tissue reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) levels were assessed. Histopathology and caspase-3 immunohistochemistry (IHC) were also performed.

Results: EEO accelerated wound closure, achieving complete healing by day 16 ($P < 0.001$). IL-1 β and TNF- α levels were reduced in EEO-treated rats (658.3 ± 52.3 pg/mg; 333.3 ± 24.72 pg/mg) compared with the control (983.2 ± 60.2 pg/mg; 650 ± 42.82 pg/mg; $P < 0.001$) and reference (841.7 ± 32.7 pg/mg; 466.7 ± 33.3 pg/mg; $P < 0.01$). CD68 decreased significantly (16.8 ± 0.9 ng/dl; $P < 0.001$ vs. control). EEO enhanced GSH and SOD and reduced ROS and MDA ($P < 0.01$). Histology showed improved re-epithelialization, granulation, and angiogenesis. Caspase-3 staining indicated controlled apoptosis.

Conclusion: EEO significantly enhances wound healing, possibly by modulating oxidative stress, inflammation, and apoptosis, thereby surpassing the efficacy of conventional therapy.

Implication for health policy/practice/research/medical education:

This study shows that Elemi essential oil (EEO) significantly accelerates wound healing by reducing inflammation, oxidative stress, and excessive apoptosis. EEO outperformed the standard silver sulfadiazine treatment, indicating its superior therapeutic potential. The findings suggest that EEO can serve as a natural, multi-targeted wound-healing agent. These results support its future development into topical formulations. Further preclinical and clinical studies are warranted to validate its translational applicability.

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Introduction

Healing wounds is an important biological process that is needed to restore skin integrity and get things back to normal after an accident. It entails a meticulously orchestrated sequence of molecular, biochemical, and cellular interactions, propelled by inflammatory mediators, growth factors, and extracellular matrix remodelling proteins (1). Disruptions in this process, whether due to infection, chronic inflammation, metabolic disorders,

or oxidative stress, significantly impair tissue repair and contribute to delayed wound healing (2). Wound-related complications present a significant global challenge, placing considerable strain on healthcare systems and financial resources. This highlights the pressing necessity for efficient, safe, and accessible therapeutic approaches to improve wound treatment results (3).

Historically, the wound-healing process was categorized into three distinct yet interconnected stages: (i) the

*Corresponding author: Krishnaraju Venkatesan,
Email: kvenkatesan@kku.edu.sa

inflammatory phase, characterized by hemostasis, vasodilation, and immune activation; (ii) the proliferative phase, defined by fibroblast migration, angiogenesis, and keratinocyte-mediated epithelialization; and (iii) the remodeling phase, during which extracellular matrix deposition and collagen maturation reestablish structural integrity and tensile strength (4). While the human body possesses intrinsic regenerative mechanisms, the presence of bacterial contamination, excessive inflammation, or oxidative damage can hinder routine healing, leading to chronic wounds, excessive scarring, or infections (5).

Current treatment strategies for wound management include antimicrobial dressings, skin grafts, bioengineered tissues, and synthetic pharmacological agents (6). Among these, topical antimicrobial agents such as antibiotics, silver-based compounds, and synthetic peptides are commonly used to prevent infection-related wound complications (7). However, the rising prevalence of antibiotic resistance, concerns about cytotoxicity, and hypersensitivity reactions have fuelled an urgent need for safer, naturally derived alternatives with broad-spectrum efficacy and minimal side effects (8). Plant-derived bioactive chemicals, such as essential oils, have emerged as viable alternatives for wound care due to their diverse therapeutic capabilities, particularly antibacterial, anti-inflammatory, and antioxidant activities (9).

Unlike many other essential oils, Elemi essential oil (EEO) was specifically chosen for this study due to its unique phytochemical profile and historical therapeutic use. Elemi resin, obtained from *Canarium luzonicum* (Burseraceae family), has long been employed for its antiseptic, anti-inflammatory, and tissue-regenerative properties, suggesting its potential relevance in modern wound care. Historically, Elemi resin has been utilised for its antiseptic and anti-inflammatory attributes, and contemporary studies have validated its antifungal (10) and antibacterial (11) efficacy. Phytochemical investigations have revealed that EEO is rich in bioactive terpenes, oxygenated terpenes, and pyrazines, which are known to modulate oxidative stress, inflammation, and microbial growth—key factors influencing wound healing (12). Therefore, EEO was selected over other essential oils due to this combination of historical use, bioactive composition, and mechanistic relevance to wound repair. This study aims to evaluate the EEO's capacity for in vivo wound healing, focusing on its bioactive constituents and their role in modulating inflammatory and regenerative processes. By examining its impact on cytokine regulation, antioxidant activity, Caspase-3 expression, and inflammatory markers, the research seeks to establish EEO as a viable natural treatment for wound care and skin tissue regeneration.

Materials and Methods

Experimental animals

In a typical laboratory setting, male Wistar rats weighing

180–200 g (3–4 months) were acquired and kept at a 12-hour light/dark cycle (Temperature: 25 ± 2 °C; RH: 55%). A typical pellet and access to water were provided to the animals. Before starting the trials, a one-week acclimatisation phase was observed. The King Khalid University Institutional Animal Ethics Committee (IAEC) gave ethical clearance for the experimental operations (Protocol No: ECM/2021-5306) (12).

Essential oil source

The oil was obtained from Organic Zing (Batch No: EO-S-24070190, Date: 07/24), New Delhi, India. According to the manufacturer's statement, the oil was produced from the resins of *Canarium luzonicum* using the steam distillation method. It was packed with pure, natural, and undiluted ingredients that met organic standards.

Identification of phytochemicals in EEO by gas chromatography-mass spectrometry (GC-MS)

The phytochemicals of EEO were examined utilising a Shimadzu GC-MS-QP2010 Plus equipment (Shimadzu Corporation, Kyoto, Japan). Separation was conducted using an HP-5MS capillary column (30 m × 0.25 mm internal diameter × 0.25 µm film thickness; Agilent Technologies, Santa Clara, USA). To accurately detect the volatile components in the oil, the analytical conditions were adjusted. Hexane 1:10 (v/v) was used to dilute the material before injection, and contaminants were removed by filtering it through a 0.22 µm membrane. Employing a split ratio of 40:1 and an injector temperature of 250 °C, 1.0 µL of the prepared sample was administered. Helium (carrier gas), maintaining a steady flow rate (1.0 mL/min). Commencing at 50 °C and maintaining that temperature for 1 minute, the oven temperature was increased by 8 °C per minute until reaching 280 °C (2 minutes). The total duration was 31 min. Ionisation was performed using electron impact (70 eV), with the ion source temperature set to 200 °C. Mass spectra were acquired within a *m/z* range of 40 to 900. The NIST Library entries (v.8; Gaithersburg, USA) were analysed against the obtained mass spectra to ascertain the compound identity.

Preparing ointments

Soft paraffin was purchased from a nearby pharmacy, and 10% w/w of EEO was blended into it to create ointment compositions. To achieve a consistent and smooth consistency, the levigating procedure was applied to a flat slab. Soft paraffin served as the reference compound, while 1% w/w silver sulfadiazine ointment acted as a positive control for comparative evaluation.

Toxicity studies

The study was conducted in accordance with OECD Guideline 404 to evaluate skin irritation (13), using 10 healthy male Wistar rats (weighing 250 to 300 g). The rats were divided into two groups, each consisting of

five animals. EEO was administered to the experimental group, whereas the other group served as the control. A test site with a diameter of about 20 cm was created by shaving the fur on the lower dorsal region with a sterile razor. Following shaving, each rat was placed in its own home and given a whole day to acclimatise without any interruptions. The experimental group's shaved region was thereafter uniformly coated with a 10% solution of the essential oil. For an hour, gauze covered with non-irritating adhesive tape was used to cover the skin with the extract. The skin was gently cleansed with distilled water after exposure, the gauze was taken off, and the area was checked for inflammation. Site observations were made 24 hours after the application, followed by additional observations 48 and 72 hours later. Rats in the control group received sterile water topically, which was administered by saturating sterile cotton with the appropriate amount of sterile water. The cotton was wrapped in gauze and non-irritating tape. Oedema and erythema were evaluated using the Draize grading scale. A score of 0 indicated no erythema or oedema, while a score of 1 reflected minimal erythema or oedema. A score of 2 indicated mild edema with slight elevation of the skin at the edge areas. A score of 3 denoted moderate to severe oedema or erythema, whereas a score of 4 indicated severe erythema or oedema (14).

Experimental plan

Eighteen male Wistar rats were assigned to three groups ($n = 6$ per group). The treatment group received a 10% w/w EEO formulation to evaluate its therapeutic effect. The reference group was treated with 1% silver sulfadiazine as a standard comparator. The control group received only soft paraffin, serving as a baseline for comparison. All treatments were applied under identical conditions to ensure consistency and allow for reliable assessment of the outcomes.

Induction of wounds following the Morton and Malone protocol

Before wound induction, the animals were fasted for 12 hours to minimise the risk of aspiration and reduce anaesthetic-related complications during intraperitoneal administration. Following the fasting period, anaesthesia was induced using ketamine (50 mg/kg) and xylazine (10 mg/kg) to ensure adequate sedation and analgesia before the surgical procedure. After the administration of anaesthesia, the animals were positioned prone on a sterile surgical platform. To preserve aseptic conditions, the dorsal surface was shaved and disinfected with 70% ethanol. An excisional wound was generated according to the technique outlined by Morton and Malone (1972). A circular, full-thickness skin wound of size approximately 2 cm in diameter was carefully excised from the dorsal thoracic region using a sterile surgical blade under semi-aseptic conditions. This specimen included epiderm,

derm, and hypodermic tissue. Sterile gauze was used to absorb extra blood. The wound was left unprotected, and during the investigation, it remained uncovered. Animals were rigorously observed for infection symptoms, and those displaying such signs were segregated and removed from the study. The animals were housed separately (15). Animals were provided with weighed feed daily at 8:00 AM. The remaining feed was evaluated the following morning to ascertain daily feed consumption.

Treatment procedure

Both the test formulation and reference drug were applied topically once daily using a sterile spatula, and treatment continued until complete epithelialization was observed. This was performed daily, with particular attention given to preventing fluctuations in dosage. Before administering therapies, the wound surfaces were meticulously washed with tampons soaked in physiological serum. The rate of contraction was measured using a transparent graph paper technique, with wound area measurements taken on days 1, 4, 8, 12, 16, and 21. The moment the eschar detached without leaving any remaining raw wound was deemed epithelization. Using CO₂ inhalation and cervical dislocation, the animals were mercifully put to death at the conclusion of the study in compliance with the AVMA Guidelines for the Euthanasia of Animals (2020). The healed lesion and adjacent skin, obtained on day 21, were removed and later divided into two equal segments for histological investigation and antioxidant measurement procedures.

Assessments

Contraction of wounds %

An excision wound was created on the dorsal thoracic region of each rat after shaving and disinfecting the area with 70% ethanol. Under mild anaesthesia (ketamine 80 mg/kg and xylazine 10 mg/kg, intraperitoneally), a circular wound of approximately 2 cm in diameter was made using a sterile surgical blade. Haemostasis was achieved with sterile gauze, and the wound was left open. Digital photographs of each wound were taken at a consistent time gap, and wound contraction was monitored by measuring the wound area on days 1, 4, 8, 12, 16, and 21 using the graph paper method. Wound areas were subsequently analysed using Adobe Photoshop and ImageJ software to ensure accurate quantification of wound contraction (16). The wound contraction percentage was calculated as follows:

$$\text{Wound contraction\%} = [(\text{Wound area on day 0} - \text{Wound area on a specific day}) / \text{Wound area on day 0}] \times 100$$

Study of biochemical markers

Collection of biological samples

Blood was collected from each group to evaluate the concentrations of CD68, IL-1 β , and TNF- α . Sampling

was performed through the orbital sinus using plain tubes, following established protocols (17). The blood was allowed to coagulate at ambient temperature following collection. The serum was then separated by centrifuging at 3,000 rpm (15 minutes). The transparent supernatant was meticulously transferred and preserved at -80°C until subsequent biochemical analysis. Separated serum was used in the experiment.

Assessment of IL-1 β and TNF- α in serum

Serum levels of IL-1 β and TNF- α were measured using commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits, following the protocols specified by the manufacturers. Using standard curves, cytokine concentrations were calculated and reported (18). The kits were specifically designed to work with serum samples, so values were interpreted straight from the standard curves without the need for normalisation to total protein concentration.

Rat serum CD68 quantification

CD68 levels in serum were quantified using a specific ELISA kit, following the manufacturer's instructions. The levels of the macrophage marker CD68 were determined using a standard calibration curve and reported (19). As with the cytokine assays, serum samples were used directly for analysis without normalization to total protein, in accordance with the kit's validated protocol.

Assessment of oxidative stress biomarkers

In a glass homogeniser, one millilitre of physiological saline was used for every gram of skin tissue. The final homogenates underwent 30 minutes of centrifugation at $10,000 \times g$ and 4°C . The clear supernatant was stored at -80°C for further analysis. The manufacturer's instructions were adhered to when employing ELISA kits (Andy Hua Tai, Beijing, China) to quantify the levels of reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) (20,21).

Histopathology of the wound site

Collection of biological samples

Samples of the dorsal skin were taken from every animal and preserved in buffered formalin. The tissues were then treated using xylene and alcohol at varying ratios before being embedded in paraffin wax. Haematoxylin and eosin (H&E) were used to stain paraffin-embedded tissue blocks after they had been sectioned at $4\text{ }\mu\text{m}$ thickness. After that, the stained sections were inspected and photographed using a Leica light microscope equipped with the Leica Application Suite (Leica Microsystems, Germany).

Immunohistochemical staining

Immunohistochemical staining for caspase-3 was performed to evaluate apoptosis in wound tissues using the

Anti-Caspase-3 antibody [EPR18297] (Abcam; by Santa Cruz Biotechnology, USA). Visualisation was achieved with DAB chromogen, followed by counterstaining with haematoxylin. A characteristic brownish-yellow colouration identified apoptotic cells. For quantification, five defined fields from three tissue sections per animal were analysed. Using Image-Pro Plus 6.0 software, the number of caspase-3-positive cells was quantified. The ratio of cells that were positively stained to all the cells in the examined fields was used to calculate the percentage of apoptotic (caspase-3 $^{+}$) cells (22).

Analysis of statistics

All data were expressed as mean $\pm$ SEM. After a one-way ANOVA for group comparisons, Tukey's multiple comparison test was used. GraphPad Prism (v.8) was used for statistical analysis, and differences were considered significant at $P < 0.05$.

Results

Chemical characterisation of essential oil

The GC-MS analysis of EEO revealed a complex composition of bioactive compounds, with 31 constituents exhibiting peak areas greater than 0.2% (Table 1). As shown in Figures 1 and 2, the study indicated that terpenes represent the principal class of chemicals, with Bicyclo[2.2.1]heptane, 7,7-dimethyl-2-methylene (7.35%), 3,6-dimethyl-1,5-heptadiene (4.74%), and 1,3,7-Octatriene, 3,7-dimethyl-, (E) (1.79%) being the most prevalent. Notably, Benzene, 1-methyl-3-(1-methylethyl)- (12.46%), an aromatic compound, was the most abundant constituent. Furthermore, oxygenated sesquiterpenes and oxygenated terpenes were identified, notably Guaiol (2.96%) and Trans-limonene oxide (0.53%), recognised for their anti-inflammatory and antioxidant properties. A pyrazine derivative, 2-methyl-3-cis-209 propenylpyrazine (4.96%), was also detected, contributing to the potential bioactivity of the oil.

Percentage of wound contraction

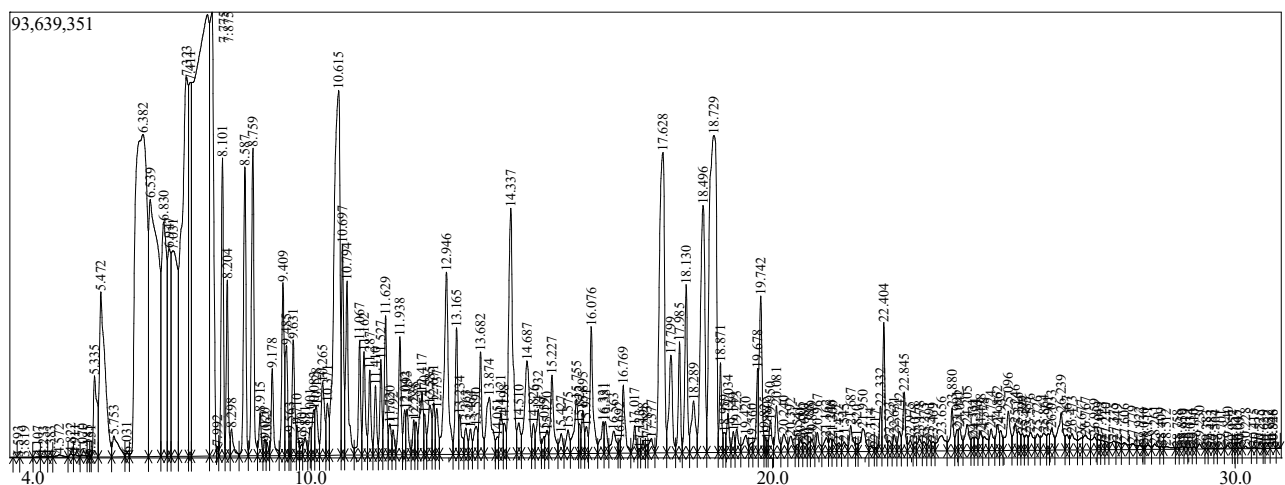
The results show that the wound healing duration of the experimental groups differed noticeably from one another (Figure 3). Complete wound closure was found in the control & reference groups only after 19 days, indicating a protracted healing phase. In contrast, animal wounds administered EEO demonstrated a significantly expedited healing process, achieving complete closure within 16 days, underscoring the oil remarkable therapeutic efficacy (Figure 4).

Body weight

The EEO-treated group showed complete wound healing, along with a significant increase in body weight (Figure 5) and feed intake (Figure 6) when compared to the other two groups. The variations observed in body weight were directly related to feed consumption.

Table 1. Elemi essential oil (EEO)-GC-MS compounds with a peak area percentage greater than 0.2%

Retention time (min)	Identified compounds	Peak area %	Molecular formula	Compound classification
5.335	Bicyclo [3.1.0] hex-2-ene, 2-methyl-5-(1-methylethyl)-	0.57	C ₁₀ H ₁₆	Terpene
5.472	1,3,7-Octatriene, 3,7-dimethyl-, (E)-isomer	1.79	C ₁₀ H ₁₆	Terpene
5.753	Bicyclo [2.2.1] heptane, 2,2-dimethyl-3-methylene-	0.25	C ₁₀ H ₁₆	Terpene
6.382	Bicyclo [2.2.1] heptane, 7,7-dimethyl-2-methylene-	7.35	C ₁₀ H ₁₆	Terpene
6.539	3,6-Dimethyl-1,5-heptadiene	4.74	C ₉ H ₁₆	Terpene
6.830	1,3-Cyclohexadiene, 2-methyl-5-(1-methylethyl)-	2.27	C ₁₀ H ₁₄	Terpene
6.947	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	1.36	C ₁₀ H ₁₄	Terpene
7.031	Bicyclo [3.1.0] hex-2-ene, 2-methyl-5-(1-methylethyl)-	2.42	C ₁₀ H ₁₆	Terpene
7.323	2-Methyl-3-cis-propenylpyrazine	4.96	C ₈ H ₁₀ N ₂	Pyrazine
7.411	Bicyclo [2.2.1] heptane, 2-(1-methylethenyl)-	1.90	C ₁₀ H ₁₆	Terpene
7.775	Benzene, 1-methyl-3-(1-methylethyl)-	12.46	C ₁₀ H ₁₄	Aromatic
7.875	Bicyclo [4.1.0] heptane, 7-(1-methylethylidene)-	3.38	C ₁₀ H ₁₆	Terpene
8.101	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	1.31	C ₁₀ H ₁₆	Terpene
8.204	1-Octanol	0.67	C ₈ H ₁₈ O	Alcohol
8.587	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	1.40	C ₁₀ H ₁₆	Terpene
8.759	1,6-Octadien-3-ol, 3,7-dimethyl-	1.52	C ₁₀ H ₁₈ O	Alcohol
9.178	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	0.41	C ₁₀ H ₁₆ O	Alcohol
9.409	4-Isopropenyl-1-methyl-7-oxabicyclo[4.1.0]heptane	0.85	C ₁₀ H ₁₆ O	Oxygenated terpene
9.485	Trans-limonene oxide	0.53	C ₁₀ H ₁₆ O	Oxygenated terpene
10.083	2-Ethyl-5-methylfuran	0.23	C ₇ H ₁₀ O	Furan
10.128	7-Oxabicyclo [4.1.0] heptane, 3-oxiranyl-	0.28	C ₇ H ₁₀ O	Epoxide
10.265	Terpinen-4-ol	0.49	C ₁₀ H ₁₈ O	Alcohol
10.371	Trans-3(10)-Caren-2-ol	0.26	C ₁₀ H ₁₆ O	Alcohol
10.615	3-Cyclohexene-1-methanol, α,α, 4-trimethyl-	4.24	C ₁₀ H ₁₈ O	Alcohol
10.697	Cyclohexanol, 1-methyl-4-(1-methylethylidene)-	0.89	C ₁₀ H ₁₈ O	Alcohol
10.794	1,3-Cyclohexadiene, 2-methyl-5-(1-methylethyl)-, monoepoxide	0.92	C ₁₀ H ₁₆ O	Epoxide
17.628	Guaiol	2.96	C ₁₅ H ₂₆ O	Oxygenated sesquiterpene



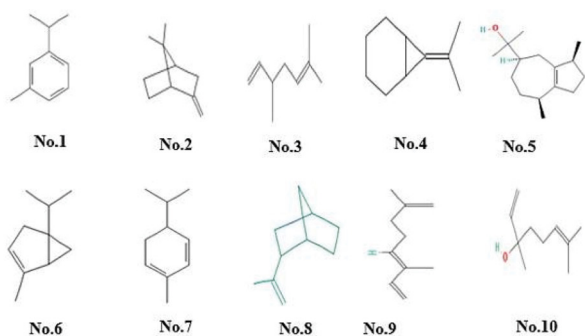


Figure 2. The major constituents identified in the Elemi essential oil. These compounds are as follows: **No. 1:** Benzene derivative – 1-methyl-3-(1-methylethyl)-benzene; **No. 2:** Bicyclic hydrocarbon–Bicyclo[2.2.1]heptane with 7,7-dimethyl-2-methylene substitution; **No. 3:** Acyclic diene – 3,6-Dimethyl-1,5-heptadiene; **No. 4:** Bicyclo[4.1.0]heptane with a 7-(1-methylethylidene) moiety; **No. 5:** Guaiol (a sesquiterpene alcohol); **No. 6:** Bicyclo[3.1.0]hex-2-ene with 2-methyl-5-(1-methylethyl) group; **No. 7:** 1,3-Cyclohexadiene with a 2-methyl-5-(1-methylethyl) substitution; **No. 8:** Bicyclo[2.2.1]heptane bearing a 2-(1-methylethenyl) group; **No. 9:** A linear diene – 1,7-Octatriene, 3,7-dimethyl-, (E)-isomer; **No. 10:** Oxygenated monoterpene – 1,6-Octadien-3-ol, 3,7-dimethyl-.

Figure 5 illustrates how feed intake increased progressively in the EEO group, showing statistically significant differences from the other groups across all days. However, the Control group exhibited the least improvement, and the Reference group experienced a significant increase.

EEO wound healing acceleration by modulating inflammatory pathways

On day 21, serum analysis revealed distinct differences in

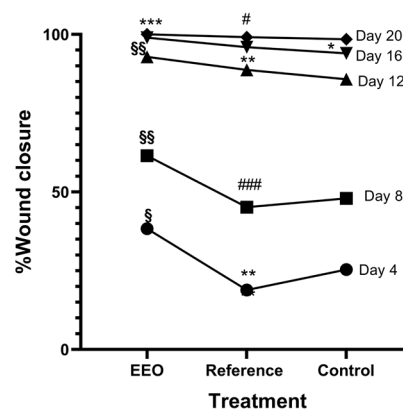


Figure 4. Effect of Elemi essential oil (EEO) on percentage wound closure in rats over 20 days. ($n = 6$). The significance for group comparisons is as follows: ### $P < 0.001$ – EEO vs. Reference; ** $P < 0.05$ – EEO vs. Reference; \$\$ $P < 0.01$ – EEO vs. Control; *** $P < 0.001$ – EEO vs. Control; # $P < 0.05$ – EEO vs. Reference; * $P < 0.05$ – Reference vs. Control; § $P < 0.05$ – EEO vs. Control.

proinflammatory cytokine levels among the experimental groups (Table 2). In the EEO-treated group, TNF- α and IL-1 β levels were 33.33 ± 24.72 pg/mg and 658.3 ± 52.31 pg/mg, respectively. The reference group showed TNF- α (466.7 ± 33.33 pg/mg) and IL-1 β (841.7 ± 32.70 pg/mg), whereas the control group exhibited TNF- α (650 ± 42.83 pg/mg) and IL-1 β (983.2 ± 60.2 pg/mg). These values indicate a marked reduction in both cytokines following EEO treatment compared with the other groups.

EEO-treated CD68 level in experimental groups

CD68, a glycoprotein expressed on macrophages, is a

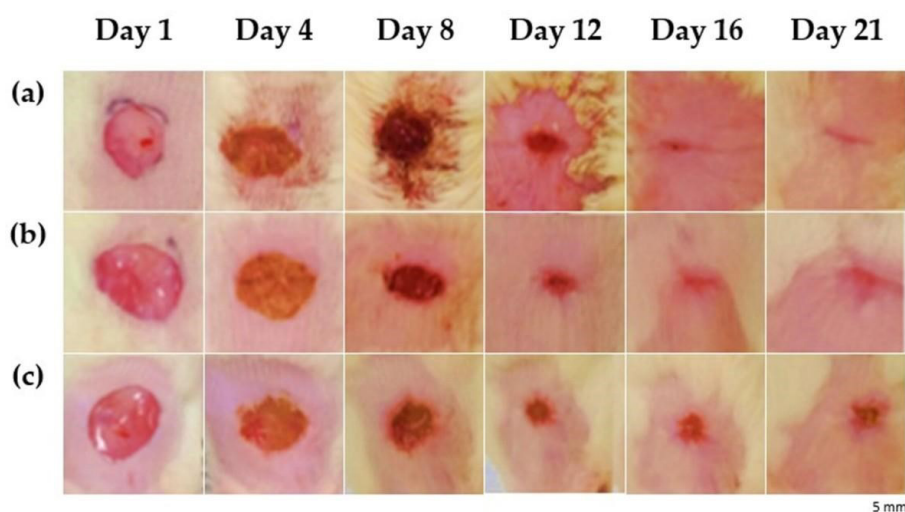


Figure 3. Macroscopic evaluation of wound healing progression over 21 days. Sequential photographic images show wound closure in three experimental groups: (a) EEO-treated, (b) Reference, and (c) Control. Photographs were taken on Days 1, 4, 8, 12, 16, and 21 post-wounding. On Day 1, uniform full-thickness excision wounds (2 cm in diameter) were created on the dorsal region of each rat using a sterile circular stencil and surgical scissors, ensuring consistent wound size and depth across all groups. Wound areas were measured using graph paper and analysed digitally using ImageJ software to ensure reproducibility and accuracy. By day 4, wound contraction and scab formation were evident in all groups. The EEO-treated group (a) demonstrated accelerated wound closure, reduced scab formation, and early tissue regeneration. By day 21, the EEO-treated wounds appeared nearly healed with minimal scarring, indicating superior efficacy compared to the other groups. Scale bar: 5 mm.

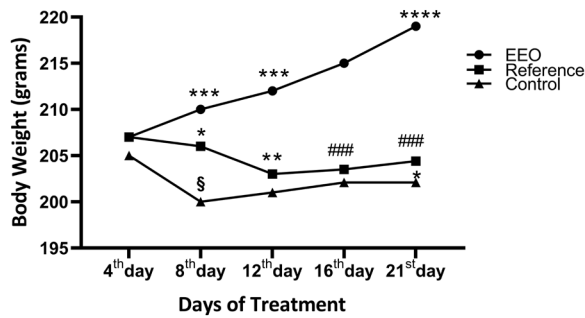


Figure 5. Effect of Elemi essential oil (EEO) on body weight over the days of treatment in rats over 21 days (n = 6 per group). The significance for group comparisons is as follows: § $P < 0.05$: Reference vs. control; * $P < 0.05$: EEO vs. reference; ** $P < 0.01$: EEO vs. Reference; ### $P < 0.001$: EEO vs. Reference; *** $P < 0.001$: EEO vs. control; and **** $P < 0.0001$: EEO vs. control.

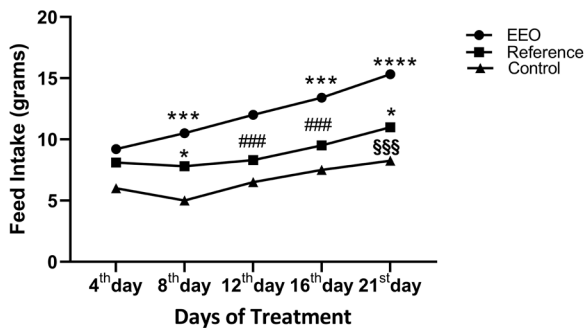


Figure 6. Effect of Elemi essential oil (EEO) on feed intake against the days of treatment in rats over 21 days (n = 6 per group). The following significance levels were observed: * $P < 0.05$ – EEO vs. reference; *** $P < 0.001$ – EEO vs. control; ### $P < 0.001$ – EEO vs. Reference; §§§ $P < 0.001$ – Reference vs. control; and **** $P < 0.0001$ EEO vs. control.

key indicator of the inflammatory response during tissue repair. Table 2 presents the levels of CD68, interleukin-1 β (IL-1 β), and tumour necrosis factor-alpha (TNF- α) in wound tissue across all experimental groups. The control group exhibited markedly elevated CD68 levels (31.83 ± 1.014 ng/dL) compared to both the reference-treated (21.33 ± 1.563 ng/dL) and EEO-treated (16.83 ± 0.910 ng/dL) groups. Similarly, IL-1 β and TNF- α levels were highest in the control group, moderately reduced in the reference-treated group, and lowest in the EEO-

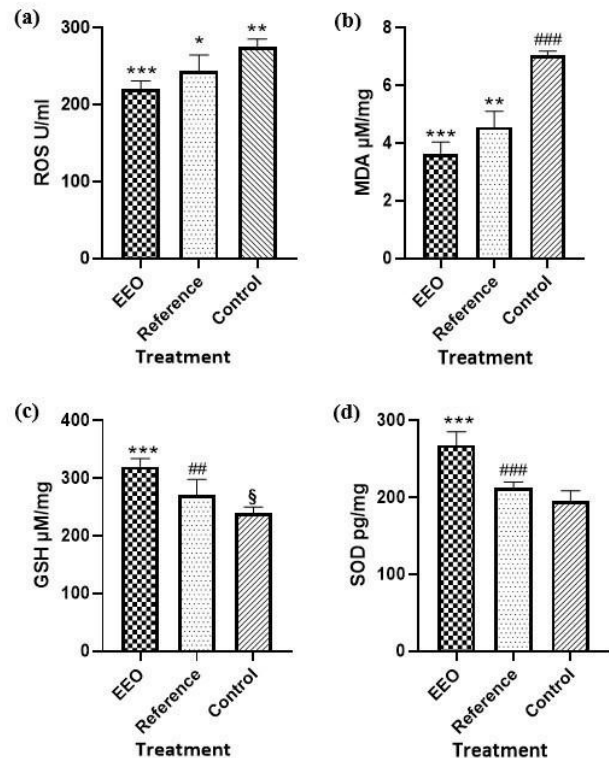


Figure 7. Effect of Elemi essential oil (EEO) on antioxidant responses on experimental groups (Day 21). The following significant differences were observed: a) ROS- * $P < 0.01$ – EEO vs Reference; ** $P < 0.01$ – Reference vs Control; *** $P < 0.001$ – EEO vs Control; b) MDA- ### $P < 0.001$ – Reference vs Control; *** $P < 0.001$ – EEO vs Control; ** $P < 0.01$ – EEO vs Reference; c) GSH- § $P < 0.05$ – EEO vs Reference; ## $P < 0.01$ – EEO vs Reference; *** $P < 0.001$ – EEO vs Control; and d) SOD- ### $P < 0.001$ – EEO vs Reference; *** $P < 0.001$ – EEO vs Control (n = 6 in each group).

treated group. Notably, the EEO-treated group showed a significantly greater reduction in all three inflammatory markers compared with the reference-treated group, indicating potent anti-inflammatory activity through macrophage modulation.

Alterations in antioxidant responses in EEO-treated rats

Marked differences in oxidative stress and antioxidant markers (ROS, MDA, GSH, and SOD) were observed among the experimental groups (Figure 6). By day 21, the EEO-treated rats exhibited significantly reduced ROS levels (220.8 ± 4.17) and MDA concentrations (3.63 ± 0.17) compared with the reference group (244.2 ± 8.41 ;

Table 2. Levels of CD68 (ng/dL), interleukin-1-beta (IL-1 β) (pg/mg), and tumour necrosis factor-alpha (TNF- α) (pg/mg) in wound tissue of experimental groups at day 21

Treatment	CD68	IL-1 β	TNF- α
EEO	$16.83 \pm 0.9098^{***}$	$658.3 \pm 52.31^{***}$	$333.3 \pm 24.72^{***}$
Reference	$21.33 \pm 1.563^{\#}$	$841.7 \pm 32.70^{*}$	$466.7 \pm 33.33^{*}$
Control	$31.83 \pm 1.014^{###}$	983.3 ± 60.09	$650 \pm 42.82^{**}$

The observed significance levels between groups are as follows: *** $P < 0.001$ – EEO vs. Control; ### $P < 0.001$ – Reference vs. Control; ** $P < 0.01$ – Reference vs. Control; * $P < 0.05$ – EEO vs. Reference; § $P < 0.05$ – EEO vs. Reference (n = 6 per group).

4.56 ± 0.22) and the control group (275.0 ± 4.28; 7.05 ± 0.05). In contrast, antioxidant parameters were markedly increased in the EEO group, with GSH levels of 320.0 ± 5.77 and SOD activity of 268.3 ± 7.03, relative to the reference (270.0 ± 11.55; 212.5 ± 3.10) and control groups (239.2 ± 4.55; 195.0 ± 5.63). These findings indicate that EEO treatment effectively restored redox homeostasis by lowering oxidative stress markers and enhancing endogenous antioxidant defence mechanisms (Figure 7).

Histopathological results

The histological changes linked to wound healing were evaluated using a semi-quantitative scoring system. Each tissue specimen was evaluated for essential healing criteria, including granulation tissue formation, collagen deposition, presence of inflammatory cells, angiogenesis, and re-epithelialization. Table 3 and Figure 8 represent

Table 3. The impact of Elemi essential oil (EEO) on histopathological changes in experimental groups (Day 21)

Histopathological features	Score		
	EEO	Reference	Control
Angiogenesis	1	2	3
Collagen deposition	1	1	1
Inflammatory response	2	1	1
Granulation tissue	2	1	1
Re-epithelialization	3	2	1

Histopathological alterations were semi-quantitatively assessed for key wound healing parameters (n= 6 per group).

consolidated histological scores and micrographs, respectively.

Immunohistochemical staining

These beneficial effects could enhance wound healing by

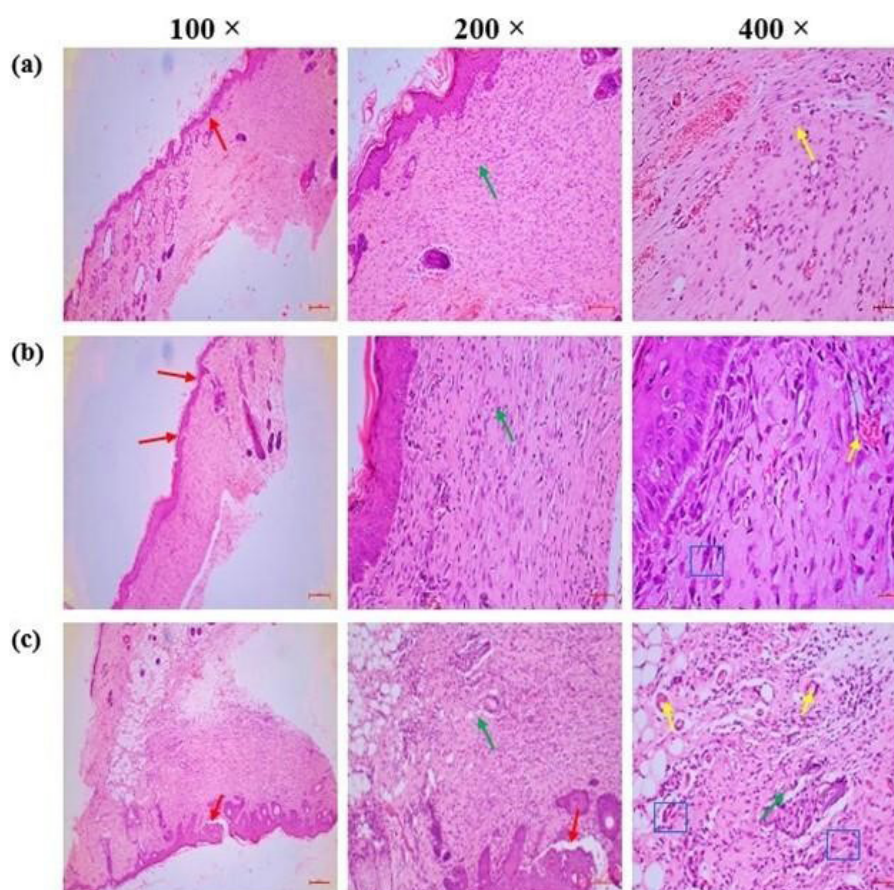


Figure 8. Histopathological observations of wound tissue in various groups (Day 21). **(a) EEO-Treated:** Complete reformation of the epidermal layer was evident (red arrow), accompanied by well-organised dermal architecture. A mild infiltration of inflammatory cells was noted (green arrow). The wound area was primarily replaced with granulation tissue rich in newly formed capillaries (yellow arrow), indicating active neovascularization. **(b) Reference:** Re-epithelialization was complete, although focal areas showed epidermal thickening (red arrow). Moderate inflammatory infiltration was observed, along with the development of granulation tissue in the dermis (green arrow). Angiogenesis was present (yellow arrow). The granulation tissue contained fibroblasts (blue square), actively involved in synthesizing collagen and extracellular matrix proteins (black arrow), as well as primarily plasma cells and lymphocytes, with occasional neutrophils. **(c) Control:** Wound healing remained incomplete, with partial epidermal coverage (red arrow) and accumulation of inflammatory exudate and necrotic debris. In the dermal region, the green arrow indicates a mild, immature inflammatory response in the granulation tissue. Moderate angiogenic activity was detected (indicated by the yellow arrow). The granulation tissue also showed fibroblasts (blue square) involved in the formation of the extracellular matrix. The EEO-treated wounds exhibited superior healing with enhanced angiogenesis and tissue organisation compared to other groups.

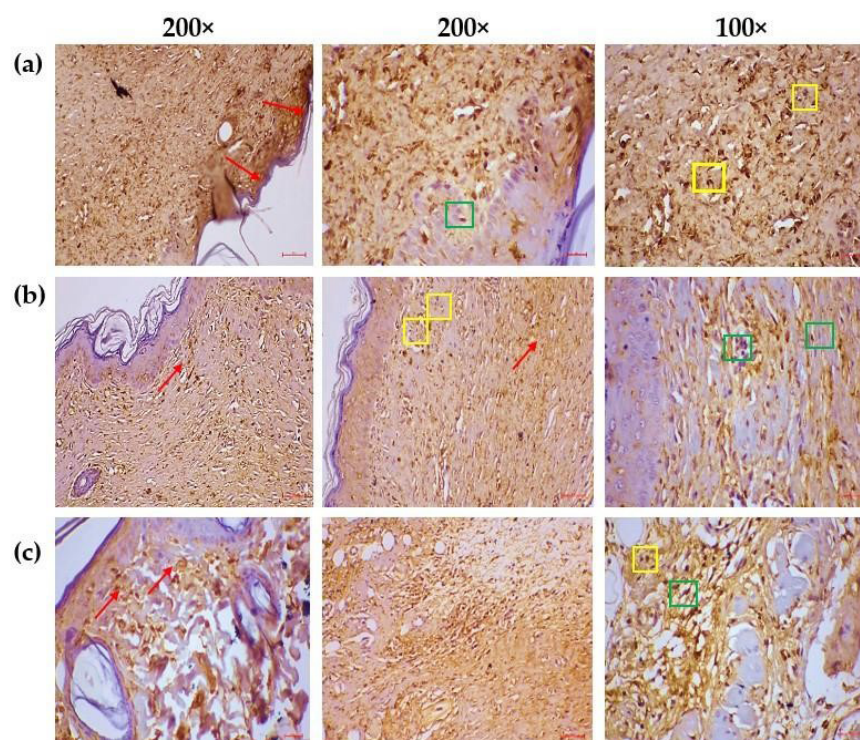


Figure 9. Caspase-3 expression in wound tissue (Day 21). (a) EEO treatment- caspase-3 expression appeared normal in the fully repaired epidermal and dermal regions (red arrow). In the granulation tissue, nucleocytoplasmic localization of caspase-3 was observed predominantly in fibroblasts (green square) and immune cell populations, such as plasma cells, lymphocytes, and neutrophils (yellow square), within both the dermal and epidermal layers. (b) Reference: Caspase-3 activity shows moderate evidence in the completely healed epidermal and dermal tissues (red arrow). A comparable level of nucleocytoplasmic staining was observed in the granulation tissue, with expression noted in fibroblasts (green square) and various immune cells, including lymphocytes and neutrophils (yellow square), distributed throughout the dermal and epidermal compartments. (c) Control: Expression of Caspase-3 appeared mild in the healed areas of the epidermis and dermis (red arrow). Granulation tissue, characterized by limited activation in fibroblasts (green square) and immune cell populations (yellow square), suggests reduced apoptotic activity in the skin layers. EEO treatment maintained optimal caspase-3-mediated apoptosis, promoting effective wound remodelling compared to other groups.

optimising cell turnover and fostering tissue regeneration, as demonstrated in Figure 9. The results indicate that EEO administration regulates caspase-3 expression in a manner conducive to effective wound repair through controlled apoptosis.

Discussion

Wound healing is a complex natural procedure including four overlapping stages (23). The utilisation of natural products like EEO has attracted considerable interest due to their potential bioactive qualities, including anti-inflammatory, antibacterial, and antioxidant activities (24). This study examined the beneficial impacts of EEO on wound healing through various parameters, including wound closure percentage, feed intake, body weight, histopathological changes, cytokine modulation, antioxidant activity, and re-epithelialization.

The GC-MS analysis of EEO revealed a diverse chemical composition, with benzene (12.46%), 1-methyl-3-(1-methyl-ethyl) (7.35%), and Guaiol (2.96%) as the major constituents. These compounds belong to the categories of monoterpenes, sesquiterpenes, oxygenated terpenes, and aromatic hydrocarbons, which contribute to the oil's

therapeutic properties. Interestingly, this study identified a higher proportion of aromatic hydrocarbons and oxygenated sesquiterpenes compared to previous reports, which predominantly highlighted *Limonene* (55.88%), *α -Phellandrene* (21.8%), and *Elemol* (10.1%) as dominant compounds (25,26). This suggests potential chemotypic variations influenced by geographical origin or extraction methods. The chemical diversity of EEO could account for its varied biological activities in wound healing.

EEO exhibited progressive wound healing. Body weight and feed intake are critical indicators of systemic recovery, as metabolic stress during injuries can lead to appetite suppression and delayed healing. The monoterpenes, aromatic hydrocarbons, and pyrazines in EEO may have contributed to stress reduction and appetite stimulation (27,28). Certain pyrazine derivatives, such as tetramethylpyrazine (TMP), have been reported to improve lipid metabolism (29) and reduce hepatic lipid accumulation (30) in experimental models, suggesting their potential role in metabolic regulation (29,30). Together, these effects produce the ideal systemic milieu for tissue regeneration and wound healing.

Wound closure percentage was a direct measure of

healing progress (31). EEO-treated wounds demonstrated significantly higher wound closure rates than controls. The bioactive components of EEO, such as Guaiol, were known to promote cellular proliferation and migration, which supports faster wound healing (32).

The antibacterial properties of EEO are extensive, affecting both Gram-positive and Gram-negative bacteria, as well as specific fungal species (33). These effects can reduce microbial load in wounds, minimising the risk of infection and accelerating healing. Notably, its terpenoid components, such as α -pinene and β -pinene, disrupt bacterial cell walls (34,35). Combining EEO with conventional antimicrobial dressings may amplify its efficacy and reduce reliance on synthetic antibiotics.

Inflammation is a crucial step in wound healing, regulated by cytokines including IL-1 β and TNF- α (36). EEO significantly downregulates the expression of IL-1 β and TNF- α , thereby preventing excessive inflammation and promoting a controlled immune response. Monoterpenes and sesquiterpenes in EEO likely contributed to this effect by inhibiting NF- κ B signalling, which acts as a key transcription factor driving the production of pro-inflammatory cytokines (37). Pyrazines play a role in suppressing NF- κ B-mediated cytokine expression, reducing IL-1 β and TNF- α levels (38). Furthermore, pyrazines decrease macrophage infiltration, as indicated by the reduction in CD68 expression, facilitating the transition from the pro-inflammatory (M1) to anti-inflammatory (M2) macrophages, which was essential for wound resolution and tissue repair (39). EEO significantly influenced this transition, as evidenced by an increase during the initial stages of healing, followed by a decrease as the wounds progressed to later phases.

Moderate levels of ROS were essential for cellular communication and pathogen defence; however, excessive oxidative stress results in fibroblast apoptosis, lipid peroxidation, and compromised wound healing (40,41). EEO exhibited significant antioxidant activity, evidenced by elevated GSH and SOD expression and reduced MDA and ROS levels. An imbalance between antioxidants and ROS leads to oxidative stress, which can hinder wound healing by damaging cellular components (42,43). Monoterpenes, recognised as free radical scavengers, are essential in mitigating oxidative stress by increasing nuclear factor erythroid 2-related factor 2 (Nrf2), the principal regulator of antioxidant defence (44). Nrf2 activation increases the synthesis of heme oxygenase-1 and other phase II detoxifying enzymes, as well as GSH-S-transferases, thereby mitigating oxidative stress-induced damage (45). Monoterpenes and sesquiterpenes (e.g., α -pinene, Limonene, and Myrcene) help stabilize cellular redox homeostasis by modulating SOD activity, thereby preventing oxidative damage (46). Moreover, pyrazines have potent antioxidant capabilities by neutralising reactive oxygen species and augmenting cellular antioxidant defence systems (47). Pyrazines also stimulate

the Nrf2 pathway, resulting in the increased production of essential antioxidant enzymes (48).

Histopathological alterations, which included keratinocyte migration and proliferation to cover the wound bed, were indicative of proper wound healing. EEO significantly accelerated re-epithelialization, as observed in histological analyses. Its antimicrobial and anti-inflammatory effects likely contributed to a favourable microenvironment for keratinocyte activity (49). Furthermore, the antioxidant properties of EEO protected epithelial cells from oxidative damage by exhibiting vigorous antioxidant activity, mitigating oxidative stress through monoterpenes (e.g., Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methyl ethyl)-) and sesquiterpenes (e.g., Guaiol), which stabilised cellular redox homeostasis by modulating GSH and SOD activity (50). Additionally, pyrazines (e.g., 2,5-dimethylpyrazine) scavenged reactive oxygen ROS and could have upregulated the Nrf2 pathway, enhancing SOD expression, protecting cells, and accelerating wound healing (51).

Apoptosis is significantly influenced by caspase-3, a vital process that helps eliminate damaged or infected cells during wound healing (52). While direct evidence connecting EEO to caspase-3 expression in wounds was limited, EEO may still have an indirect effect on apoptotic pathways by reducing oxidative stress and inflammation, two factors known to influence caspase activation (53). Monoterpenes and oxygenated terpenes in EEO may help modulate caspase-3-dependent apoptosis, preventing excessive cell death in tissues while supporting tissue regeneration (54). These beneficial effects could enhance wound healing by optimising cell turnover and fostering tissue regeneration. Through its regulating influence on inflammation and oxidative stress, EEO has been shown in this study to have a positive effect on caspase-3.

The present study was conducted in an animal model, which may not completely represent the complex wound-healing mechanisms in humans. Molecular markers such as VEGF, TGF- β 1, and Nrf2 were not assessed, which restricted mechanistic insight. Dose response and formulation stability were also not assessed. Hence, further mechanistic and clinical investigations are needed to validate the therapeutic efficacy and safety of EEO in wound management.

Conclusion

The present study underscores the multifaceted wound-healing potential of EEO, demonstrating its efficacy as a natural therapeutic agent. EEO not only enhanced systemic health parameters, such as body weight and feed intake, but also accelerated wound contraction and promoted complete tissue regeneration. These effects appear to be mediated through the coordinated modulation of key cellular and molecular mechanisms, including balanced caspase-3 activity, suppression of TNF- α and IL-1 β , enhanced macrophage activity (as indicated by CD68

expression), and restoration of antioxidant homeostasis. Together, these mechanisms may have contributed to re-epithelialization, organised dermal architecture, and increased cellular proliferation, highlighting the superior healing outcomes achieved with EEO compared to control and reference treatments.

While the findings provide compelling evidence for EEO's therapeutic potential, further studies are warranted to expand its applicability. Investigations into multiple dosages, additional wound models, and deeper molecular pathway analyses would strengthen understanding of the precise mechanisms involved and support clinical translation. Such studies could provide valuable insights into optimising EEO-based wound therapies and confirm their efficacy across diverse biological systems.

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Authors' contribution

Conceptualization: Yahya I. Asiri.

Data curation: Yahya I. Asiri, Krishnaraju Venkatesan.

Formal analysis: Yahya I. Asiri, Krishnaraju Venkatesan.

Funding acquisition: Yahya I. Asiri.

Investigation: Yahya I. Asiri, Krishnaraju Venkatesan.

Methodology: Yahya I. Asiri, Krishnaraju Venkatesan.

Project administration: Yahya I. Asiri, Krishnaraju Venkatesan.

Resources: Yahya I. Asiri, Krishnaraju Venkatesan.

Software: Yahya I. Asiri, Krishnaraju Venkatesan.

Supervision: Yahya I. Asiri, Krishnaraju Venkatesan.

Validation: Yahya I. Asiri, Krishnaraju Venkatesan.

Visualization: Yahya I. Asiri, Krishnaraju Venkatesan.

Writing—original draft: Yahya I. Asiri, Krishnaraju Venkatesan.

Writing—review & editing: Yahya I. Asiri, Krishnaraju Venkatesan.

Conflict of interests

The authors declare that there was no potential conflict of interest in this paper.

Data availability statement

Available on request.

Ethical considerations

The Institutional Animal Ethics Committee (IAEC) at

King Khalid University (IAEC) (ECM/2021-5306/ Dated 02 May 2021) approved all experimental procedures.

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