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# Wound healing properties, antimicrobial and antioxidant activities of *Salvia kronenburgii* Rech. f. and *Salvia euphratica* Montbret, Aucher & Rech. f. var. *euphratica* on excision and incision wound models in diabetic rats☆

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ABSTRACT

Diabetic patients suffer from persistent and non-healing wounds. *Salvia* species are traditionally used for the treatment of wounds and colds. The aims of the present study were to evaluate the *in vivo* wound healing potential, *in vitro* antimicrobial and antioxidant activities, and total phenolic and flavonoid contents of the aerial parts of two endemic taxa, *Salvia kronenburgii* Rech. f. (SK) and *Salvia euphratica* Montbret, Aucher & Rech. f. var. *euphratica* (SE). Two different concentrations (0.5% and 1% (w/w)) of ethanol extracts were investigated in incision and excision wound models on Streptozotocin-induced diabetic rats using biomechanical, biochemical, histopathological, macroscopic, and genotoxic methods for 7 and 14 days. Antimicrobial activity was evaluated against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Acinetobacter baumannii*, *Aeromonas hydrophila*, *Mycobacterium tuberculosis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* using the broth microdilution and the resazurin microtiter assay plate methods. Fito®, Ampicillin, Ethambutol, Isoniazid, and Fluconazole were used as reference drugs. Antioxidant capacities and total phenolic and flavonoid contents of both extracts were detected using DPPH free radical scavenging assay, Folin-Ciocalteu, and $Al(NO_3)_3$ methods, respectively. SK ointment at 0.5% and 1% (w/w) concentrations and SE ointment at 1% (w/w) concentration showed 99.9%, 99.5%, and 99.7% contraction, respectively for excision wounds, and SK and SE ointments at 1% (w/w) concentration showed 99.4% and 99.2% contraction for incision wounds while Fito® showed 98.9% and 98.5% contraction, respectively. Increased re-epithelialization ($P < 0.01$ and $P < 0.001$), angiogenesis, and decreased dermal inflammation ($P < 0.001$) were determined for SK and SE ointments at both 7 and 14 days. SE ointment on day 7 and SK ointment on day 14 reduced oxidative damage to DNA when compared to control ($P < 0.01$ and $P < 0.001$). Both tested plants had greater antibacterial activity against *A. baumannii* (62.5 µg/mL MIC value) and SE had greater antimycobacterial activity against *M. tuberculosis* (0.24 µg/mL MIC value) when compared to reference drugs Ampicillin, Isoniazid, and Ethambutol (125, 0.97, and 1.95 µg/mL MIC values, respectively). Antioxidant capacities, total phenolic and flavonoid contents of SE and SK were 87.08%, 76.21 µg GAE/mg, 43.43 µg QE/mg and 72.17%,

*Abbreviations:* DM, *Diabetes mellitus*; SK, *Salvia kronenburgii*; SE, *Salvia euphratica* var. *euphratica*; STZ, streptozotocin; EtOH, ethanol; DMSO, dimethyl sulfoxide; NOx, nitric oxide; TBA, thiobarbituric acid; MDA, malondialdehyde; GSH, glutathione; H&E, hematoxylin and eosin; VVG, Verhoeff-Van Gieson; MN, micronucleus; GDI, genetic damage index; DCP, damaged cell percent; MNPCEs, micronucleated polychromatic erythrocytes; REMA, resazurin microtitre assay; MIC, minimum inhibitory concentration; MMC, mitomycin-C; TPC, total phenolic content; TFC, total flavonoid content; DPPH, 2,2-diphenyl-1-picrylhydrazyl; BHA, butylated hydroxyanisol; GA, gallic acid; GAE, gallic acid equivalent; QE, quercetin equivalent

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41.81 µg GAE/mg, 33.62 µg QE/mg, respectively. SK and SE had strong wound healing effects while SK found to be more effective than SE at both 7 and 14 days.

## 1. Introduction

*Diabetes mellitus* (DM) is one of the most common diseases leading to disability and death worldwide with increasing prevalence [1]. Public health research predicts that the number of adults with diabetes will increase to nearly 439 million by 2030 [2]. In the United States, 15% of 29.1 million diabetic patients suffer from serious complications of DM including chronic ulcers commonly known as persistent, impaired, and/or non-healing wounds [3] which significantly reduce their quality of life [4]. Multifactorial reasons including hyperglycemia, disruption of homeostasis, inhibition of inflammatory response, generation of reactive oxygen species, reduction in collagen production, poor angiogenesis, dysfunction in the differentiation of the extracellular matrix, and fibroplasia disturb the healing process in diabetic wounds [3].

Although there are many synthetic drugs using for wound treatment, expense and adverse effects limit their role in the management of wounds. Therefore, alternative treatment approaches are needed. The rich source of medicinal plants available at a cheap cost are one such strategy [5].

*Salvia* L. (commonly known as sage) is one of the largest genus of plants in the Lamiaceae (formerly Labiatae) family which comprises nearly 1000 species distributed in different regions of the world [6,7]. The Latin word *salvare,* which means "to be safe and unharmed or to heal", is thought to be the origin of the genus name and this word translates to *sauge* (sage) in French [7]. In the old world, Anatolia is an important diversity center of *Salvia* [8] and in Turkish flora, the genus is represented by 100 species, 53 of which are endemic [9]. Since ancient times various traditional uses of *Salvia* species, including treatment of stomach ailments [6,10], menstrual disorders [10,11], tuberculosis, bronchitis [7], wounds [9], hepatitis, hemorrhage [11], the common cold [6,10], microbial infections, cancer, inflammation, and malaria [7], have been reported. Additionally, treatment of wounds [9], cold and cough [12], and antibacterial, antiseptic [9], diuretic, carminative, spasmolytic [12], and stimulant [9] properties of *Salvia* species (many of them are named as adaçayı) are well known in Turkish folk medicine. Herbal teas [9], food preservatives [13], flavoring agents in cosmetic and perfumery industries [10], and aromatherapy [7,10] are other important uses of *Salvia* species.

The presence of biologically active secondary metabolites such as flavonoids, phenolic acids, and terpenoids (especially abietane-type diterpenes) [12] have been reported for the genus. In addition to these components, the essential oil compositions of *Salvia* species are rich in oxygen-containing sesquiterpenes, monoterpene hydrocarbons, and oxygen-containing monoterpenes [7]. Anti-inflammatory [7,10,13], antiproliferative, immunomodifying, antineurodegenerative [13], antioxidative [7,13], wound healing [14–16], antibacterial, antifungal [10], antiviral, antitubercular, cytotoxic [9], cardioprotective [13], antiprotozoal [12], and insecticidal [7] activities of various *Salvia* species have been investigated in previous studies.

*Salvia kronenburgii* Rech. f. and *Salvia euphratica* Montbret, Aucher & Rech. f. var. *euphratica* are endemic taxa growing in Turkey [17]. A review of the literature data revealed no studies on wound healing or antimicrobial activity of the ethanol extracts of these two taxa. Therefore, the aim of this study was to evaluate wound healing, anti(myco) bacterial, antifungal, antioxidant effects, and total phenolic and flavonoid contents of ethanol extracts obtained from aerial parts of *S. kronenburgii* (SK) and *S. euphratica* var. *euphratica* (SE).

## 2. Materials and methods

### 2.1. Plant material

SK and SE were collected from their natural habitats in the Eastern Anatolian Region of Turkey. Identification and confirmation of the samples were performed by Dr. Ahmet Kahraman, Department of Biology, Faculty of Arts and Science, Uşak University (Uşak, Turkey). The dried voucher specimens were stored in the Plant Systematics and Phylogenetics Research Laboratory, Uşak University. Specimen details are provided in Table 1.

### 2.2. Extraction procedure

The powdered air-dried aerial parts of the plant material were macerated three times with ethanol (EtOH, 96%, Merck, Darmstadt, Germany) at room temperature (500 mL of EtOH per 100 g of plant material). Whatman Grade No.1 filter paper was used for the filtration procedure. After filtration, solvents were evaporated using a vacuum evaporator (Heidolph-Rotar TLR 1000) at 35–40 °C and plant extracts were stored in the dark at 4 °C until studied.

### 2.3. In vivo wound healing activity

#### 2.3.1. Ointment processing

A simple ointment base was prepared with glycol stearate:propylene glycol:liquid paraffin at a ratio of 3:6:1 according to Suntar et al. [15]. For plant test ointments (0.5% and 1% (w/w)) appropriate amounts of each plant extract were added to the simple ointment base. The vehicle group was topically treated with the simple ointment base alone. Fito® cream (Tripharma Drug Industry and Trade Inc., Turkey) containing 15% (w/w) *Triticum vulgare* L. aqueous extract was used as a reference drug. The wound areas were treated with 0.5 g of the ointments or reference drug topically once daily depending upon group assignment throughout 7 or 14 days.

#### 2.3.2. Animals and experimental protocol

A total of 84, healthy male Wistar albino rats 3- to 4-months old, weighing 180 to 240 g, was used for the study. The animals were obtained from the Research Center of Experimental Animals, Mersin University, Mersin, Turkey. The individually housed animals were kept in cages under standard conditions (light/dark cycle of 12 h/12 h with 50–70% humidity, at 25 °C ± 3 °C) and fed a standard pellet diet and water *ad libitum* during the study (7 or 14 days). The experimental protocol was approved by the Ethics Committee of Mersin University School of Medicine (Reg. No. 2016/05). The animals were randomly divided into seven groups of twelve animals with six animals from each group treated for 7 days and six for 14 days. The experimental design of the study is detailed in Table 2.

**Table 1**
Details of studied plants *Salvia kronenburgii* and *Salvia euphratica* var. *euphratica*.

| | Studied Taxa | |
|---|---|---|
| | *Salvia kronenburgii* | *Salvia euphratica* var. *euphratica* |
| Locality | Van province, Van to Gürpınar, slopes, 38.36434 °N - 43.39092 °E | Malatya province, Darende to Gürün, 12 km from Darende, hilly steppe and calcareous rocky slopes, 38.60481 °N - 37.46690 °E |
| Altitude (m) | 2072 | 1450-1500 |
| Collection date | 28.06.2015 | 02.07.2015 |
| Voucher reference | A. Kahraman 2112 | A. Kahraman 2130 |
| Phytogeography | Irano-Turanian element | Irano-Turanian element |
| Extract Yield (%) | 7.25 | 6.74 |

*2.3.3. Induction of DM*

DM was induced by using a single (45 mg/kg) intraperitoneal injection of STZ (Sigma Chemicals Co., USA) freshly prepared in saline [18]. Blood glucose levels were measured three days after STZ application using a rapid glucometer (Bayer, Germany). Rats with levels above 300 mg/dL were considered diabetic.

*2.3.4. Anesthesia*

Xylazine hydrochloride (Rompun®; Bayer, Germany) and ketamine hydrochloride (Ketanest®; Pfizer Inc., USA) were used as anesthetics and injected intraperitoneally at doses of 10 mg/kg and 30 mg/kg, respectively [19].

*2.3.5. Wound models*

All surgical procedures were performed under anesthesia. The dorsal aspect of each anesthetized animal was shaved, and the area cleaned and sterilized with ethanol (70% (v/v)).

*2.3.5.1. Excision wound model:*. For the evaluation of wound contraction and wound closure time, an open excision-type wound was initiated using a 1.5 cm diameter biopsy punch on the dorsal interscapular region of the rats with removal of the skin. After the surgical procedure the excisional wounds were left open [20]. Test ointments of SK and SE (0.5% and 1% (w/w)), reference drug Fito®, or vehicle were topically applied once a day throughout 7 or 14 days. On day 8, six randomly selected animals in each group were euthanized. The remaining six animals in each group continued to be treated topically with test ointments, reference drug, or vehicle for 14 days. On day 15, the remaining animals were euthanized.

*2.3.5.2. Incision wound model:*. For the evaluation of biomechanical parameters, an incision-type wound model 4 cm in length was created (2 cm posterior to the excision-type wound) for which three surgical sutures were placed 1 cm apart [15]. Test ointments of SK and SE (0.5% and 1% (w/w)), reference drug Fito®, or vehicle were topically applied once a day throughout 7 or 14 days. On the 7$^{th}$ post-wound day, in each group the sutures of six randomly selected animals were removed and on day 8 these animals were euthanized. The remaining six animals in each group were treated topically with test ointments, reference drug Fito®, or vehicle for 14 days. On the 14$^{th}$ post-wound day, the sutures were removed and on day 15, these animals were euthanized.

At the end of the two application periods (7 and 14 days) tissue samples were excised for further examination.

*2.3.6. Macroscopic study*

Under anesthesia, wounds were created and subsequently photographed with a camera (Spot Insight QE, Diagnostic Instruments, USA). On day 0 operated rats were individually housed. After 7- and 14- day treatments, wounds were re-photographed. Evaluation of wound areas on days 0, 7, and 14 were performed with the SPOT Advanced (Diagnostic Instruments) program to determine wound contraction. Briefly, the length and surface of the incision and excision wound areas were measured using graph paper 7 and 14 days after wound creation (day 0) and a wound healing ratio was calculated for each animal using the following formula: (%) = 100 × (1- specific day wound size/initial wound size) [20].

*2.3.7. Biomechanical study*

Incision wound samples were biomechanically tested using a Tensile Testing System (Ilfa Electronic San.Tic.Ltd., Turkey). Briefly, an automatic action clamp was used to attach each skin sample to the cross heads of a computer-controlled Tensile Testing System. Using a 1000 N load cell, crosshead speed was adjusted from 0 to 250 mm/min. Skin samples were placed between two clamps of equal distance. Clamped skin was pulled to rupture at a crosshead speed of 25 mm/min [21,22]. The data was digitized, displayed, and saved in a Tensile Test System computer. A load-deformation curve was then recorded using the tensile test system software (Ilfa Electronic San.Tic.Ltd., Turkey). Subsequently, the saved data was evaluated with a Logger Pro Software Programme (V 3.8.3, Vernier Software & Technology, Orlando, FL, USA). The biomechanical parameters (the maximum load ($F_U$: N), the maximum deformation (du: mm), the energy stored until yield point (U: mJ) and stiffness (S: N/mm)) were acquired according to the load-deformation curve. The load-deformation curve was converted to a stress-strain curve and the following parameters were calculated: the maximum strain ($\varepsilon_U$: mm/mm), maximum stress (σu: MPa), young's (elasticity) modulus (E: MPa) and toughness (u: MPa).

*2.3.8. Biochemical study*

*2.3.8.1. Measurement of hydroxyproline levels:*. Weighed samples were frozen, lyophilized and pulverized then dissolved in 1 mL of isopropyl alcohol (50% (v/v)) and hydrolyzed. Of this hydrolysate 25 µL were mixed with chloramine-T. After 10 min, 1 mL of Ehrlich's reagent was added and the mixture was incubated for 90 min at 50 °C After the reaction, color change measurements were performed using a spectrophotometer (Shimadzu UV 1601, Shimadzu, Tokyo, Japan) at 560 nm. A different concentration series of hydroxyproline standards (1.6, 1.2, 0.8, 0.6, 0.4 and 0.2 µg) were studied under the same conditions. Concentrations of all tested samples were calculated according to a standard curve. Results are provided as µg/mg dry weight of tissues [23].

*2.3.8.2. Measurement of nitric oxide levels:*. Measurement of nitric oxide (NOx) levels were performed according to Bories and Bories [24] which was briefly based on conversion of nitrate to nitrite using nicotinamide adenine dinucleotide phosphate (NADPH) with the existence of nitrate reductase. The nitrite reacts with N-(1-Naphthyl) ethylene diamine and sulfanomide and the end product absorbance calculated at 540 nm. Wound tissues were weighed, diluted with phosphate buffered saline (PBS) (pH: 7.4) and homogenized at a ratio of 1/5 (w/v). The homogenates were centrifuged (10.000 × *g*) at 4 °C for 20 min. Nitrate-nitrite assay kits (Cayman Chemical, USA) were used for measurement of tissue NOx levels at 540 nm and the results are provided as µM.

**Table 2**
Experimental design for diabetic rat wound treatment groups (N = 84).

| Group code | Group name | Treatment | Group size (n)* |
|---|---|---|---|
| Control | Control | None = negative control | 12 |
| Vehicle | Vehicle | Simple ointment base (glycol stearate:propylene glycol:liquid paraffin (3:6:1)) | 12 |
| Fito | Fito® cream | Fito® cream = positive control | 12 |
| SK-0.5 | 0.5% (w/w) *Salvia kronenburgii* ointment | 0.5% (w/w) *S. kronenburgii* ointment | 12 |
| SK-1 | 1% (w/w) *S. kronenburgii* ointment | 1% (w/w) *S. kronenburgii* ointment | 12 |
| SE-0.5 | 0.5% (w/w) *Salvia euphratica* var. *euphratica* ointment | 0.5% (w/w) *S. euphratica* var. *euphratica* ointment | 12 |
| SE-1 | 1% (w/w) *S. euphratica* var. *euphratica* ointment | 1% (w/w) *S. euphratica* var. *euphratica* ointment | 12 |

* For each group n = 6 for 7-day applications and n = 6 for 14-day applications.

*2.3.8.3. Measurement of malondialdehyde levels:*. The thiobarbituric acid (TBA) test was used for determination of lipid peroxidation end product-malondialdehyde (MDA) levels. The TBA test was performed according to the spectrophotometric measurement of the concentration obtained from the end product of the reaction between TBA and lipid peroxides [25]. Wound tissues were weighed, diluted in RIPA buffer and then homogenized at a ratio of 1/5 (w/v). After centrifugation of the homogenates (1.600 × *g*, 10 min, 4 °C), TBARS assay kits (Cayman Chemical, USA) were used to measure tissue MDA levels at 530 nm and all results are provided as nmoles/g tissue.

*2.3.8.4. Measurement of glutathione levels:*. Determination of tissue glutathione (GSH) levels were studied according to the method of Ellman [26]. Briefly, the number of aliphatic thiol groups in the tested sample were quantified using Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) = DTNB) which reacts with tested tissue at a mild alkali pH after which p-nitrophenol anion per every thiol group was calculated spectrophotometrically. Wound tissues were weighed, diluted in 50 mM metaphosphoric acid (pH: 6–7, containing 1 mM EDTA) and homogenized at a ratio of 1/5 (w/v) then centrifuged (10.000 × *g*, 15 min, 4 °C). GSH assay kits (Cayman Chemical, USA) were used for measuring the tissue GSH levels at 410 nm. All results are provided as nmoles/mg protein.

*2.3.8.5. Measurement of protein levels:*. The tissue protein levels were measured according to the Bradford technique, which is fast and sensitive for protein binding, and the results shown as micrograms (μg) [27].

*2.3.9. Histopathologic study*

At the end of the experiments (on days 7 or 14), collected skin tissues were fixed in formaldehyde (10%) and embedded in paraffin wax. Sections were cut approximately 5 μm thick, deparaffinized, then stained using hematoxylin and eosin (H&E) [28], a reticulin stain kit (Atom Scientific, UK) for reticulin fibers [29], and a Verhoeff-Van Gieson (VVG) stain kit (Atom Scientific, UK) [30] for elastic fibers. Samples were examined under a light microscope (Olympus, Japan) and scored by a blind observer. Wound healing was evaluated in terms of re-epithelialization, thickness of granulation tissue, angiogenesis, extent of dermal inflammation, presence of fibrosis, and amount of early collagen present.

Parameters used in assessment of healing score were as follows, for re-epithelialization: 0 = no re-epithelialization, 1 = mild < 50% of the wound, 2 = moderate > 50% of the wound, and 3 = complete re-epithelialization [31]; for angiogenesis: 0 = no angiogenesis, 1 = mild < 5 vessels per 1 high power field, 2 = moderate 6–10 vessels per 1 high power field, and 3 = marked > 10 vessels per 1 high power field; for dermal inflammation: 0 = no dermal inflammation, 1 = mild in superficial dermis, 2 = moderate in reticular dermis, and 3 = marked involving subcutaneous fat tissue; for granulation tissue thickness: 0 = none, 1 = scant, 2 = moderate, and 3 = marked; for amount of early collagen: 0 = none, 1 = scant, 2 = moderate, and 3 = marked [32]; and for absence or presence of fibrosis: 0 and 1, respectively. A normal distribution featuring data (Shapiro-Wilk test, $p \geq 0.05$) were analyzed by parametric test ANOVA (post-hoc: Tukey's HSD) for multiple comparisons. P values ≤ 0.05 were approved as statistically significant.

*2.3.10. Genotoxicologic study*

Genotoxic studies were performed in rat peripheral blood samples using comet [33] and micronucleus (MN) [34] assays after 7 and 14 days of treatment.

*2.3.10.1. The comet assay:*. At the end of experiments blood samples were taken from the heart. RPMI 1640 Medium (RPMI, Capricorn Scientific GmbH, Germany) supplemented with 10% Fetal Bovine Serum (FBS, Capricorn Scientific GmbH, Germany) was used for washing (x2) isolated blood cell suspensions. Isolated cell samples were then directly used for the assay based on the method performed under alkaline conditions mentioned by Singh et al. [33] with some modifications. Frozen microscopic slides were covered with 0.5% normal melting agarose (NMA, Sigma Chemicals Co., USA) dissolved in $Mg^{2+}$ and $Ca^{2+}$ -free PBS (Capricorn Scientific GmbH, Germany) at nearly 50 °C A blood suspension of 100 μL was diluted with PBS (1 mL) in Eppendorf tubes set in a water bath at 40 °C and 30 μL of the mixture was stirred with 250 mL of Low Melting Agarose (LMA, Sigma Chemicals Co., USA) (0.5%). The prepared mixture (100 μL) was put on NMA-coated slides with a micropipette and then immediately covered with coverslips. After slides were kept at 4 °C for 15 min, the coverslips were carefully removed and the slides were put into chalets containing lysis solution and stored in a dark refrigerator for 2 h. Slides were washed with ice-cold distilled water and put on a horizontal gel electrophoresis unit for filling with freshly prepared electrophoretic buffer (0.3 M NaOH + 1 mM EDTA) for 20 min to allow DNA unwinding. The electrophoresis process was then conducted at 20 °C for 20 min (25 V, 300 mA). These steps were performed in the dark to avoid DNA damage. After electrophoresis, slides were washed with ice-cold distilled water and preserved for 5 min in neutralizing buffer then washed with ice-cold distilled water again and preserved for 10 min in frozen EtOH. Ethidium bromide (0.1 mg/mL, 1:4) was used for staining of DNA. Slides were then screened using a fluorescent microscope (BX51, Olympus, Tokyo, Japan). Each test group was studied with six parallel samples. To determine genetic damage in the comet assay two different parameters were studied. Mitomycin-C (MMC) was used as a positive control.

Genetic damage index (GDI): The results provided as arbitrary units (AU) expressed the extent of DNA damage. The following equation was used to calculate results:

$N_i$: Number of scored cells in i level. i: Level of DNA damage (0, 1, 2, 3 and 4)

AU values indicating the comet assay scores were: UD (undamaged, 0); Type 1 (low damaged, 1); Type 2 (moderate damaged, 2); Type 3 (high damaged, 3); Type 4 (ultra high damaged, 4).

GDI: [(0xType 0) + (1xType 1) + (2xType2) + (3xType 3) + (4xType 4)]

Damaged Cell Percent (DCP): Type 2 + Type 3 + Type 4 [28]

*2.3.10.2. The micronucleus assay:*. Blood smears were prepared on microscope slides then air dried and fixed with methanol (Merck, Darmstadt, Germany). Acridine orange (125 mg/mL in pH 6.8 phosphate buffer) was used for staining. After stainining for 1 min the tested samples were evaluated with a fluorescence microscope [34]. A total of 2000 polychromatic erythrocytes (PCE) per animal were used to determine the number of micronucleated polychromatic erythrocytes (MNPCEs). MMC was used as a positive control. The results of the MN assay were evaluated according to color changes (immature erythrocytes, *i.e.* PCEs were identified according to their orange-red color, mature erythrocytes green color, and micronuclei yellowish color).

*2.4. In vitro antimicrobial study*

The antimicrobial activities of ethanol extracts obtained from aerial parts of SK and SE were investigated using two different methods including a broth microdilution method for antibacterial [35] and antifungal [36–38] activities and the resazurin microtiter assay (REMA) plate method for antimycobacterial activity [39].

*2.4.1. Microbial strains*

Gram-positive bacterial strains [*Staphylococcus aureus* (ATCC 25925) and *Bacillus subtilis* (ATCC 6633)], gram-negative bacterial strains [*Escherichia coli* (ATCC 25923), *Acinetobacter baumannii* (ATCC

02026) and *Aeromonas hydrophila* (ATCC 95080)], *Mycobacterium tuberculosis* H37Rv, and fungal strains [*Candida tropicalis* (ATCC 750), *Candida glabrata* (ATCC 90030) and *Candida parapsilosis* (ATCC 22019)] were purchased from the Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey.

#### 2.4.2. Antibacterial study

Antibacterial activities of the plants were studied against two gram-positive and three gram-negative bacterial strains. The broad spectrum semisynthetic penicillin Ampicillin was chosen as a reference drug. To obtain an initial concentration of 2000 µg/mL, each extract was dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) to prepare stock solutions which were diluted in Mueller-Hinton broth. Further dilutions of the tested extracts and Ampicillin were prepared at different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9 and 1.9 µg/mL) [35]. Standard strain working suspensions were prepared in sterile tubes and turbidity adjusted to match McFarland standard No: 0.5. A 1:20 dilution of each suspension was prepared using distilled water of which 10 µL was added to each plate and the bacterial concentration of each plate adjusted to $5 \times 10^5$ CFU/mL [40]. The microbial growth effects of DMSO were also studied using a control test containing inoculated broth. The same dilutions of DMSO which were prepared for the extracts and reference drug were tested and results indicated that the solvent had no effect on the studied bacteria. The minimal inhibitory concentration (MIC) values of tested extracts were determined in duplicate tests [35].

#### 2.4.3. Antimycobacterial study

For preparing culture medium, 7H9-S medium was used with Middlebrook 7H9 broth (Becton Dickinson, USA) containing 0.1% casitone, 0.5% glycerol, and 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson, USA). For preparing resazurin reagent, resazurin sodium salt powder (Sigma R7017, Sigma Chemicals Co., USA) was used. A working solution was prepared at a 0.01% (w/v) concentration in distilled water and a 0.22 µm membrane filter (Ministar, Sartorius Stedim Biotech GmbH, Goettingen, Germany) was used for filtration and sterilization procedures. The solution was stored at 4 °C. The REMA plate method mentioned in Nateche et al. [39] was performed in duplicate with some modifications. Reference drugs Ethambutol (Sigma, E4630, Sigma Chemicals Co., USA) and Isoniazid (Sigma, I3377, Sigma Chemicals Co., USA), and the standard strain *M. tuberculosis* H37Rv were used for experiments. Stock solutions of reference agents and extracts were prepared in DMSO at a 1000 µg/mL concentration and filtered through 0.22 µm membrane filters. A two-fold dilution series of all solutions was prepared with 100 µL of 7H9-S in a 96-well microtiter plate. Concentrations ranging from 0.12 to 250 µg/mL were studied. A sterility control without inoculum and a growth control containing no antibiotic were added to each plate. The H37Rv inoculum was made by resuspending a loopful of Lowenstein-Jensen culture medium in a tube containing 5 mL 7H9-S medium with several glass beads. For 2 min the tube was vortexed and then let sit for 30 min to allow sediment formation. The supernatant was put into sterile tubes and the turbidity adjusted to match McFarland standard No: 1. This prepared suspension was diluted (1:20) in 7H9-S. All plates were inoculated with 100 µL of suspension, kept in plastic bags and incubated for 7 days at 37 °C in normal atmosphere. After the incubation period, 30 µL of resazurin working solution was added to each well. The plates were then incubated again for 24 h at 37 °C. The results were reported visually. Color changes from blue to pink indicated reduction of resazurin which meant bacterial growth. For a positive result, the color changes showing growth had to be comparable to that seen in the positive growth control. The MIC value was determined as the lowest concentration of the test solution which prevented full color changes of resazurin.

#### 2.4.4. Antifungal study

The antifungal activities of the plant extracts were assessed against three fungal strains with respect to the standard document (M27-A2) of NCCLS [36]. Fluconazole (Sigma, F8929, Sigma Chemicals Co., USA) a synthetic triazole was used as a reference antifungal agent. Activity studies were performed in RPMI 1640 medium (Sigma, R6504, Sigma Chemicals Co., USA) buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS, Sigma, M1254, Sigma Chemicals Co., USA) as mentioned in the M27-A2 standard document. A working suspension of standard strains was prepared as a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 medium. Stock solutions of the reference drug and extracts were prepared at 1000 µg/mL concentration in DMSO and all prepared solutions were filtered using a 0.22 µm membrane filter. A two-fold dilution series of reference drug and prepared solutions were put in a 96-well microtiter plate using 100 µL RPMI 1640 medium. Concentrations ranging from 0.12 to 250 µg/mL were tested. A sterility control without inoculum and a growth control containing no antibiotic were added to each plate. Additionally, 100 µL of the working inoculum suspension was pipetted into each plate and whole plates were incubated for 48 h at 35 °C in ambient air. MIC values known as the lowest concentrations of the tested plant extracts that inhibited growth of the studied microorganism was visually detected [36–38].

### 2.5. Total phenolic, flavonoid contents and DPPH radical scavenging activity

#### 2.5.1. Determination of total phenolic content

The total phenolic content (TPC) of the each extract was determined by the Folin-Ciocalteu method as described by Ag Seleci et al. [41]. Briefly, 200 µL of the Folin-Ciocalteu reagent (2.0 N) was mixed with 1160 µL of distilled water and 40 µL of each extract dissolved in EtOH (2 mg/mL). After 5 min (at 25 °C), 600 µL of $Na_2CO_3$ (20%) solution was added. Subsequently, the mixture was shaken in a water bath for 30 min at 40 °C in the dark. The absorbance of the mixture was measured using a spectrophotometer (Cary 60 UV–vis, Agilent, USA) at 765 nm. The results were quantified by calibration curve obtained from measuring the standard solutions of gallic acid (GA) (50–500 µg/mL) and expressed in µg GA equivalent (GAE)/mg extract. The TPCs were calculated by the following equation which was obtained using the standard GA curve ($R^2 = 0.990$): $A_{765\ nm} = 0.0025 \times [GAE]$. Analyses were run in triplicates and the results were given as the mean ± standard deviation (SD).

#### 2.5.2. Determination of total flavonoid content

The total flavonoid content (TFC) of the each extract was measured by using the method of Moreno et al. [42] with some modifications [43]. Briefly, an aliquot of 40 µL of different concentrations of quercetin (5–100 µg/mL) solutions and 40 µL of each extract (2 mg/mL) dissolved in EtOH were added to different tubes which contained 40 µL of Al $(NO_3)_3$ (10%), 40 µL of $CH_3COOK$ (1.0 M) and 1520 µL of EtOH. The mixture was thoroughly mixed and allowed to stand at room temperature for 40 min. Then absorbance of the mixture was measured using a spectrophotometer (Cary 60 UV–vis, Agilent, USA) at 415 nm. Quercetin was used for the standard calibration curve. Results were expressed as µg quercetin equivalent (QE)/mg extract and the TFCs were calculated by the following equation which was obtained using the standard quercetin curve ($R^2 = 0.992$): $A_{415\ nm} = 0.0116 \times [QE]$. Analyses were run in triplicates and the results were given as the mean ± SD.

#### 2.5.3. DPPH radical scavenging activity

The stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the each extract was performed using the method described by Blois [44] with some modifications by Gunes et al. [45]. Butylated hydroxyanisol (BHA) was used as a reference test material. The samples and BHA dissolved in EtOH. 1 mL of the each sample (10–250 µg/mL) and BHA (2.5–250 µg/mL) were mixed with 1 mL of

DPPH solution (0.1 mM) in different test tubes. The mixtures were shaken vigorously and incubated for 30 min in the dark at room temperature. Absorbance of the each solution was measured using a spectrophotometer (Cary 60 UV–vis, Agilent, USA) at 517 nm. The DPPH radical scavenging activity (%) was calculated using the following equation: DPPH radical scavenging activity (%) = 100 x [($A_0$–$A_1$)/$A_0$)], where; $A_0$ was the absorbance of the control reaction mixture without the test material, and $A_1$ was the absorbance of the test material. Analyses were run in triplicates and the results were given as the mean ± SD.

### 2.6. Statistical analyses

The statistical analyses and bar charts were prepared using SPSS 20.0 (IBM, New York, USA), MS Office Excel (Microsoft Corporation, USA), and Graph Pad Prism 6 (GraphPad Software, USA). The data are represented as the mean ± SD and all data were statistically evaluated using one-way analysis of variance (ANOVA) with Tukey post-hoc tests. The confidence interval was set at 95%, and P values < 0.05 and < 0.01 were considered as significant. Additionally, for genotoxicity studies, normality of all data were checked with a Kolmogorov–Smirnov D test. Based upon their normal distribution (Kolmogorov–Smirnov D test, P ≥ 0.05), a Student *t*-test was applied to the parametric data. P values < 0.05 were considered statistically significant.

## 3. Results

In the present study, ethanol extracts obtained from aerial parts of two endemic *Salvia* taxa were evaluated for their total phenolic and flavonoid contents, *in vitro* antibacterial, antifungal, antimycobacterial, and antioxidant activities, and *in vivo* wound healing activities using macroscopic, biomechanical, biochemical, histopathological, and genotoxic methods on excision and incision wound models in STZ-induced diabetic rats for 7 and 14 days. Detailed information about the studied plants with extract yields are provided in Table 1.

### 3.1. Macroscopic study

During the wound healing period (day 0, 7, and 14), excision (Fig. 1) and incision skin wounds (Fig. 2) were photographed. Wound size measurements were performed with a Spot program and the wound healing ratio calculated for each animal (Table 3). In the excision wound model, after 7-day applications, wound healing ratios of SK and SE ointments (0.5% and 1% (w/w)) and Fito® cream, which was used as a positive control, were found to be statistically significant when compared to control (P < 0.01) and vehicle (P < 0.05) groups. SK-1 and SE-0.5 were found to be the most effective with wound healing ratios of 68.5% and 69.5%, respectively. Fito, SK-0.5, and SE-1 showed slightly lower efficiency at 67.5%, 65.7%, and 63.6%, respectively. After 14-day applications, wound healing ratios of SK and SE ointments (0.5% and 1% (w/w)) and Fito were found to be statistically significant when compared to control and vehicle (P < 0.05) groups. The wound healing ratios of SK-0.5, SK-1, and SE-1 at 99.9%, 99.5%, and 99.7%, respectively were slightly more effective than SE-0.5 and Fito (both 98.9%) treatments. Healing ratios of control and vehicle groups were 36.2% and 44.8% at 7 days and 73.2% and 87.5% at 14 days, respectively. For the incision wound model, after both application periods of 7 and 14 days, wound healing ratios of SK and SE ointments (0.5% and 1% (w/w)) and Fito® cream were found to be statistically significant when compared to control (P < 0.01) and vehicle (P < 0.05) groups. At both 7 and 14 days, the wound healing ratios of SK-1 were higher than all tested groups at 85.0% and 99.4%, respectively, while control and vehicle healing ratios were 29.5% and 44.2% at 7 days and 73.5% and 78.9% at 14 days, respectively (Table 3).

### 3.2. Biomechanical study

Biomechanical tests were performed on incision wound samples and results are detailed in Table 4. After 7-day applications, biomechanical parameters of control and vehicle groups were similar. The energy absorption capacity of SK-1 and SE-1 were significantly lower than Fito. Comparison of control, vehicle, and Fito showed that Fito had the statistically highest energy absorption capacity with a value of 121.82 ± 16.47 (P < 0.05). The stiffness and elasticity values of SE-0.5 were statistically significantly higher than Fito and vehicle (P < 0.05). Similar stiffness and elasticity values were observed in Fito, SK-1, and SE-1 groups. Elasticity values for SK-0.5 and SE-0.5 were higher than Fito (0.06 ± 0.02, 0.08 ± 0.03, and 0.03 ± 0.01, respectively). The maximum deformation and maximum strain values for Fito were statistically higher than control, vehicle, and SK and SE ointment (0.5% and 1% (w/w)) groups (P < 0.05). The toughness value of Fito was statistically higher than control, vehicle, and SK and SE ointment (1% (w/w)) groups (P < 0.05) as well. The maximum load and maximum stress values for SE-1 were significantly lower than Fito (P < 0.05). Increased maximum load values were observed in Fito, SK-0.5, and SE-0.5 groups (23.62 ± 3.30, 24.14 ± 7.51, and

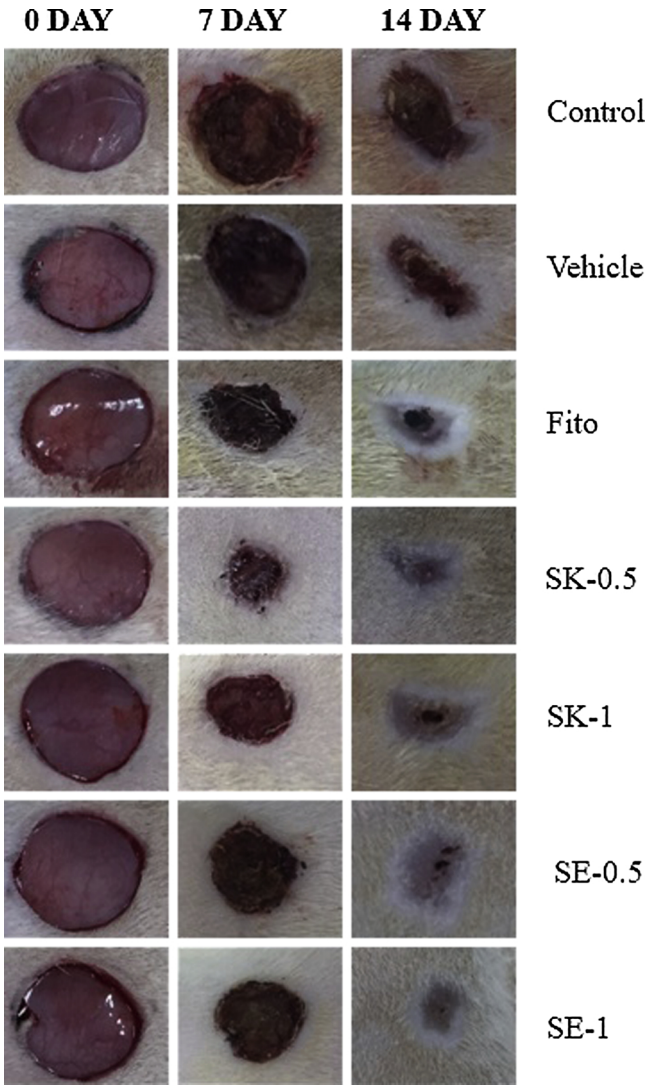


**Fig. 1.** Macroscopic view of excision wounds for 0, 7 and 14 days. (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *S. kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *S. euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

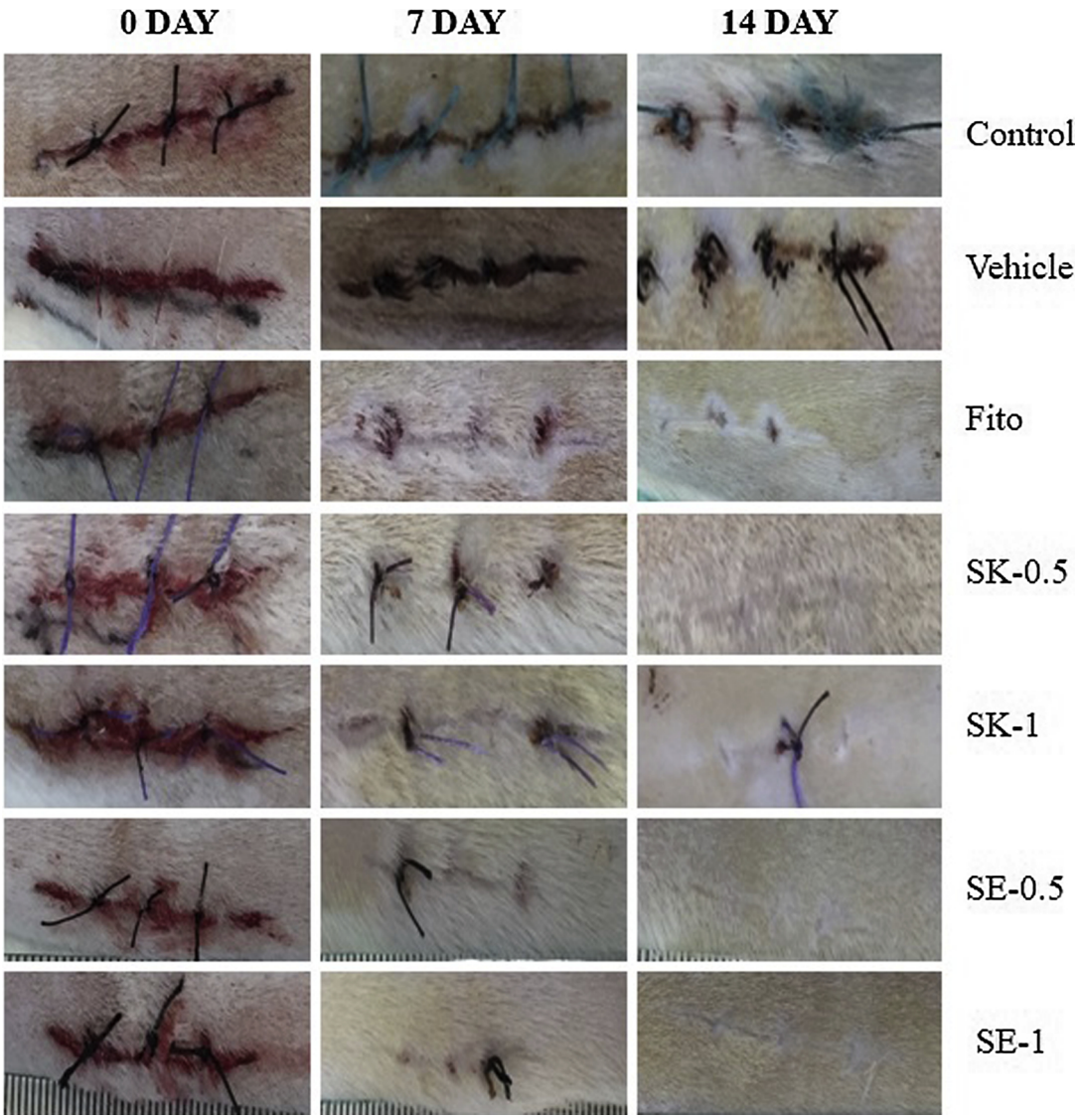


**Fig. 2.** Macroscopic view of incision wounds for 0, 7 and 14 days. (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *S. kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *S. euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

**Table 3**
Wound healing ratio (% contraction) of excision and incision wound models in diabetic rats based on treatment.

| Groups | | Control | Vehicle | Fito | SK-0.5 | SK-1 | SE-0.5 | SE-1 |
|---|---|---|---|---|---|---|---|---|
| **Excision wound model** | | | | | | | | |
| Application Periods | 7-Day | 36.2 ± 24.7[a] (5.2-65.4) | 44.8 ± 14.4[a,b] (26.3-62.8) | 67.5 ± 8.8[c] (53.8-79.5) | 65.7 ± 10.7[c] (44.9-73.7) | 68.5 ± 7.3[c] (57.1-75.8) | 69.5 ± 10.9[c] (50.1-78.4) | 63.6 ± 12.8[c] (47.4-84.8) |
| | 14-Day | 73.2 ± 20.8[a] (35.2-92.9) | 87.5 ± 10.5[a] (73.5-97.5) | 98.9 ± 1.1[b] (97-100) | 99.9 ± 0.1[b] (99.5-99.9) | 99.5 ± 0.6[b] (98.3-99.9) | 98.9 ± 1.5[b] (96.2-99.9) | 99.7 ± 0.6[b] (98.5-100) |
| **Incision wound model** | | | | | | | | |
| Application Periods | 7-Day | 29.5 ± 17.6[a] (14.6 -57.7) | 44.2 ± 23.2[a,b] (23.1-44.7) | 81.4 ± 15.9[c] (49.1-91.1) | 78.2 ± 4.9[c] (73-86.9) | 85.0 ± 5.0[c] (76.5-89.2) | 72.0 ± 8.6[c] (61.4-86.7) | 77.5 ± 17.5[c] (45.5-92.9) |
| | 14-Day | 73.5 ± 11.9[a] (56.1-88.3) | 78.9 ± 14.9[a,b] (62.9-95.4) | 98.5 ± 1.5[c] (96.3-100) | 98.1 ± 2.5[c] (94.5-100) | 99.4 ± 0.5[c] (98.7-100) | 96.9 ± 3.0[c] (93.1-100) | 99.2 ± 1.2[c] (97.1-100) |

[a] ANOVA (post-hoc: Tukey's HSD) analysis was performed ($P > 0.05$).
[b] ANOVA analysis was performed ($P < 0.05$).
[c] ANOVA analysis was performed ($P < 0.01$). Measuring the average ± SD. Min-Max value intervals are in parenthesis. (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *Salvia kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *Salvia euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

**Table 4**
Biomechanical parameters of incision wound model skin samples from diabetic rats.

| Groups | Skin Biomechanical Parameters | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Energy Absorption Capacity (U, mj) | Stiffness (S, N/mm) | Maximum Load ($F_U$: N) | Maximum Deformation (du: mm) | Young's (Elasticity) Modulus (E, MPa) | Toughness (u; MPa) | Maximum Stress (σu; MPa) | Maximum Strain ($\varepsilon_U$, mm/mm) |
| **7-Day application** | | | | | | | | |
| Control | 84.51 ± 23.90 | 4.71 ± 2.14 | 18.96 ± 5.22 | 10.47 ± 2.45 | 0.06 ± 0.03 | 0.21 ± 0.06 | 0.09 ± 0.03 | 4.23 ± 1.23 |
| Vehicle | 83.16 ± 30.90 | 3.58 ± 1.61 | 19.84 ± 3.69 | 11.06 ± 1.51 | 0.04 ± 0.02 | 0.21 ± 0.08 | 0.10 ± 0.02 | 4.53 ± 0.76 |
| Fito | 121.82 ± 16.47[a,c] | 3.20 ± 1.57 | 23.62 ± 3.30 | 15.05 ± 2.34[a,c] | 0.03 ± 0.01 | 0.31 ± 0.04[a,c] | 0.12 ± 0.02 | 6.52 ± 1.17[a,c] |
| SK-0.5 | 91.95 ± 54.11 | 5.40 ± 2.45 | 24.14 ± 7.51 | 8.67 ± 3.46[b] | 0.06 ± 0.02 | 0.23 ± 0.14 | 0.12 ± 0.04 | 3.33 ± 1.73[b] |
| SK-1 | 83.13 ± 23.55[b] | 3.50 ± 1.26 | 20.86 ± 6.08 | 10.17 ± 1.94[b] | 0.03 ± 0.01 | 0.21 ± 0.06[b] | 0.10 ± 0.03 | 4.07 ± 0.97[b] |
| SE-0.5 | 79.59 ± 40.94 | 7.81 ± 2.59[b,c] | 25.03 ± 4.03 | 7.52 ± 3.29[b] | 0.08 ± 0.03[b,c] | 0.20 ± 0.10 | 0.13 ± 0.02 | 2.76 ± 1.64[b] |
| SE-1 | 58.52 ± 16.08[b] | 3.02 ± 0.53 | 15.31 ± 1.85[b] | 8.60 ± 1.71[b] | 0.03 ± 0.01 | 0.16 ± 0.04[b] | 0.08 ± 0.01[b] | 3.30 ± 0.86[b] |
| **14-Day application** | | | | | | | | |
| Control | 173.38 ± 48.67 | 3.46 ± 2.43 | 27.07 ± 6.72 | 16.15 ± 4.71 | 0.04 ± 0.03 | 0.43 ± 0.11[b] | 0.13 ± 0.03 | 7.05 ± 2.29 |
| Vehicle | 98.04 ± 43.47 | 4.17 ± 2.76 | 25.86 ± 6.74 | 11.43 ± 3.94 | 0.04 ± 0.03 | 0.25 ± 0.12 | 0.13 ± 0.03 | 4.72 ± 1.97 |
| Fito | 172.81 ± 70.50 | 4.27 ± 2.23 | 30.34 ± 6.36 | 14.73 ± 3.93 | 0.04 ± 0.02 | 0.45 ± 0.18 | 0.15 ± 0.03 | 6.39 ± 1.97 |
| SK-0.5 | 121.02 ± 10.54 | 6.28 ± 1.76 | 35.59 ± 6.19[a] | 11.00 ± 2.73 | 0.06 ± 0.02 | 0.29 ± 0.04 | 0.18 ± 0.03[a] | 4.63 ± 1.37 |
| SK-1 | 113.57 ± 47.05 | 5.22 ± 1.89 | 28.62 ± 7.62 | 10.95 ± 3.02 | 0.05 ± 0.02 | 0.29 ± 0.13 | 0.14 ± 0.04 | 4.46 ± 1.46 |
| SE-0.5 | 71.35 ± 13.01[a,b] | 9.09 ± 3.04[a,b,c] | 28.78 ± 6.29 | 7.98 ± 2.54[b,c] | 0.09 ± 0.03[a,b,c] | 0.18 ± 0.03[a,b] | 0.14 ± 0.03 | 2.99 ± 1.27[a,b] |
| SE-1 | 85.59 ± 8.33[a] | 7.16 ± 2.44 | 28.96 ± 5.89 | 9.31 ± 2.57[a] | 0.07 ± 0.02 | 0.21 ± 0.02[a] | 0.14 ± 0.03 | 3.66 ± 1.28[a] |

Data was analyzed by parametric test ANOVA (post-hoc: Tukey's HSD).
[a] Significantly different from Control ($P < 0.05$).
[b] Significantly different from Fito ($P < 0.05$).
[c] Significantly different from Vehicle ($P < 0.05$). Measuring the average ± SD (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *Salvia kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *Salvia euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

25.03 ± 4.03, respectively). Maximum stress values for Fito, SK-0.5, and SE-0.5 ranged from 0.12 ± 0.02 to 0.13 ± 0.02. No statistically significant difference was observed between the other groups. Comparison of tested biomechanical parameters of SK and SE ointments (0.5% and 1% (w/w)) for 7 days showed that SK-0.5 and SE-0.5 ointment administration resulted in better skin quality than SK-1 and SE-1 ointment administration with higher values of energy absorpsion capacity, maximum load, elasticity, toughness and maximum stress, and lower maximum deformation.

After 14-day applications, the energy absorption capacity, toughness, and maximum strain values for SE-0.5 were statistically lower than Fito and control, while the same parameters for SE-1 were statistically lower than control ($P < 0.05$). Stiffness and elasticity with SE-0.5 were significantly higher than Fito, control, and vehicle ($P < 0.05$). Elasticity results for SK and SE ointment-treated groups were higher than in the Fito group. Maximum load and maximum stress values of SK-0.5 (35.59 ± 6.19 and 0.18 ± 0.03, respectively) were statistically increased compared to control (27.07 ± 6.72 and 0.13 ± 0.03, respectively; $P < 0.05$). Also, the maximum stress value of SK-0.5 was higher than Fito with a value of 0.15 ± 0.03. When compared to Fito and vehicle, statistically decreased maximum deformation was observed in SE-0.5 (14.73 ± 3.93, 11.43 ± 3.94, and

**Table 5**
Hydroxyproline, nitrogen oxides (NOx), malondialdehyde (MDA) and glutathione (GSH) levels in studied groups.

| Groups | Control | Vehicle | Fito | SK-0.5 | SK-1 | SE-0.5 | SE-1 |
|---|---|---|---|---|---|---|---|
| **7-Day application** | | | | | | | |
| Hydroxyproline | 0.34 ± 0.05[a] (0.27-0.39) | 0.38 ± 0.03[a,b] (0.35-0.43) | 0.52 ± 0.05[b,c] (0.44-0.58) | 0.51 ± 0.04[b,c] (0.45-0.57) | 0.50 ± 0.1[b,c] (0.36-0.7) | 0.62 ± 0.03[c] (0.58-0.67) | 0.52 ± 0.6[b,c] (0.43-0.61) |
| NOx | 23.9 ± 2.2[a,b] (20.6-26.3) | 26.5 ± 0.8[a] (25.5-27.7) | 24.1 ± 4.2[a,b] (18.7-30.3) | 16.5 ± 2.3[b,c] (13.7-19.2) | 20.9 ± 4.5[b] (13.7-25.5) | 28.7 ± 3.3[a] (25.5-34.1) | 20.8 ± 4.02[b] (25.5-34.1) |
| MDA | 15.1 ± 2.2[a] (12.9-18.2) | 11.2 ± 0.9[b] (10.6-12.9) | 12.3 ± 1.7[b] (9.4-13.9) | 8.4 ± 0.7[c] (7.6-9.5) | 7.9 ± 0.9[c] (7.1-9.7) | 8.5 ± 0.5[c] (7.9-9.3) | 8.1 ± 0.5[c] (7.1-8.5) |
| GSH | 12.8 ± 1.6[a] (11.5-15.9) | 13.06 ± 1.8[a] (11.1-15.5) | 11.27 ± 1.27[a] (9.2-12.6) | 11.26 ± 2.4[a] (7.9-15.1) | 7.6 ± 0.7[c] (6.6-8.2) | 15.5 ± 1.9[a] (12.2-17.7) | 9.3 ± 1.2[a] (7.9-10.9) |
| **14-Day application** | | | | | | | |
| Hydroxyproline | 0.43 ± 0.08[a,b] (0.36-0.59) | 0.50 ± 0.1[a] (0.35-0.64) | 0.39 ± 0.1[a,b] (0.25-0.47) | 0.33 ± 0.9[b] (0.2-0.42) | 0.33 ± 0.02[b] (0.29-0.36) | 0.37 ± 0.03[b] (0.35-0.43) | 0.34 ± 0.04[b] (0.28-0.39) |
| NOx | 15.5 ± 2.4[a,b,c] (12.2-18.8) | 15.2 ± 1.9[a,b,c] (11.9-17.6) | 18.5 ± 5.9[a,b] (12.2-27.7) | 21.8 ± 5.4[a] (15.5-29.5) | 10.9 ± 2.4[c] (8.3-15.2) | 11.6 ± 2.9[c] (9.6-15.6) | 10.3 ± 1.1[c] (9.5-12.5) |
| MDA | 11.5 ± 1.0[a] (10.5-12.9) | 11.9 ± 1.0[a] (10.5-12.9) | 7.42 ± 2.2[c] (4.5-9.9) | 7.5 ± 0.9[c] (6.0-8.4) | 5.67 ± 1.4[c] (4.4-7.9) | 7.2 ± 1.0[c] (6.3-9.0) | 4.6 ± 1.1[c] (3.7-6.7) |
| GSH | 6.9 ± 1.58[a,b] (5.4-9.6) | 6.15 ± 1.2[a] (4.2-7.9) | 7.93 ± 2.2[a,b] (5.5-10.3) | 10.01 ± 1.4[b] (7.2-11.2) | 7.2 ± 2.06[a,b] (4.4-10.36) | 7.3 ± 1.2[a,b] (5.7-8.9) | 6.2 ± 0.8[a] (5.4-7.2) |

[a] ANOVA (post-hoc: Tukey's HSD) analysis was performed ($P > 0.05$).
[b] ANOVA analysis was performed ($P < 0.05$).
[c] ANOVA analysis was performed ($P < 0.01$). Measuring the average ± SD. Min-Max value intervals are in parenthesis. (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *Salvia kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *Salvia euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

7.98 ± 2.54, respectively). Additionally, the same parameter for SE-1 was significantly lower than control (9.31 ± 2.57 and 16.15 ± 4.71, respectively; $P < 0.05$). Comparison of tested biomechanical parameters for SK ointments (0.5% and 1% (w/w)) for 14 days showed that SK-0.5 ointment administration resulted in better skin quality than SK-1 ointment administration, with higher values for energy absorpsion capacity, maximum load, elasticity, and maximum stress, while SE-1 ointment administration resulted in better skin quality than SE-0.5 ointment administration with higher energy absorpsion capacity, toughness, maximum strain, and lower stiffness. Moreover, comparison of biomechanical parameters of SK and SE for 7 and 14 days showed that SK appeared to be more effective than SE, and SK was also nearly as effective as the reference drug Fito® cream.

### 3.3. Biochemical study

#### 3.3.1. Measurement of hydroxyproline levels

After 7-day applications, SE-0.5 with the highest hydroxyproline level (0.62 ± 0.03) was found to be statistically significant when compared to control and vehicle ($P < 0.01$) groups. The hydroxyproline levels for SK-1, SK-0.5, SE-1, and Fito (range 0.50 ± 0.1 to 0.62 ± 0.03) were found to be statistically different than control (0.34 ± 0.03) ($P < 0.05$ and $P < 0.01$). After 14-day applications, hydroxyproline levels of SK and SE ointments (0.5% and 1% (w/w)) were found to be statistically lower than vehicle ($P < 0.05$). Statistically significant differences were not found between Fito and SK and SE ointment (0.5% and 1% (w/w)) groups on days 7 and 14 (Table 5).

#### 3.3.2. Measurement of NOx levels

After 7-day applications, significant differences were found with SK-0.5 which had the lowest NOx level (16.5 ± 2.3) ($P < 0.05$ and $P < 0.01$). In addition, SK-1 and SE-1 ($P < 0.05$) were found to be significantly different than SE-0.5 and vehicle. After 14-day applications, SK-1, SE-0.5, and SE-1 which had the lowest NOx levels at 10.9 ± 2.4, 11.6 ± 2.9, and 10.3 ± 1.1 values, respectively were significantly different compare to SK-0.5 and Fito ($P < 0.01$). No statistically significant differences were observed between the other studied groups ($P > 0.05$) (Table 5).

#### 3.3.3. Measurement of MDA levels

After 7-day applications, the highest MDA levels were observed in control at 15.1 ± 2.2. Significantly lower MDA levels were found in SK and SE ointment (0.5% and 1% (w/w)) groups (range 7.9 ± 0.9–8.5 ± 0.5) ($P < 0.01$) and Fito and vehicle ($P < 0.05$) groups compared to control. After 14-day applications, MDA levels for Fito, SK, and SE ointment (0.5% and 1% (w/w)) groups (range 4.6 ± 1.1–7.5 ± 0.9) were found to be statistically lower than control and vehicle ($P < 0.01$) groups (Table 5).

#### 3.3.4. Measurement of GSH levels

After 7-day applications, GSH level for SE-0.5 (15.5 ± 1.9) was significantly higher than other studied groups ($P < 0.01$). After 14-day applications, SK-0.5 with the highest GSH level (10.1 ± 1.4) was found to be statistically different than vehicle and SE-1 ($P < 0.05$) groups. There were no significant differences between the other tested groups ($P > 0.05$) (Table 5).

### 3.4. Histopathologic study

Histopathological evaluation of studied tissue sections are detailed in Table 6 and Figs. 3–5. Epithelial regeneration for Fito, SE-0.5 ($P < 0.01$) and SK-0.5, SK-1, and SE-1 ($P < 0.001$) were found to be significantly greater than control and vehicle on the 7th day. Comparison of efficiency of Fito® cream and plant extract ointments showed that 0.5% (w/w) SE was as effective as Fito® cream (re-epithelialization values: 1.5 ± 0.6 and 1.5 ± 0.5, respectively). Both 0.5% and 1% (w/w) SK ointments and 1% (w/w) SE ointment were more effective than Fito® cream with 2.0 ± 0.9, 2.1 ± 0.7, and 2.2 ± 0.9 re-epithelialization values, respectively, on day 7. Increased epithelial regeneration values for Fito, SK, and SE ointments (0.5% and 1% (w/w)) were found to be statistically significant when compared to both control and vehicle on the 14th day ($P < 0.001$). Also 0.5% (w/w) SK and SE ointments were found to be as effective as Fito® cream with 2.2 ± 0.7, 2.2 ± 0.8, and 2.2 ± 04 re-epithelialization values, respectively, while 1% (w/w) SK and SE ointments exhibited increasing re-epithelialization with 2.7 ± 0.5 and 2.3 ± 0.7 values, respectively, when compared to Fito® cream on day 14. Granulation tissue thickness ($P < 0.05$) and type III collagen ($P < 0.001$) levels of SK and SE ointment (0.5% and 1% (w/w)) groups were found to be statistically lower than Fito, control, and vehicle on the 7th day. Comparison of all

**Table 6**
Histological comparison of excision wound values per treatment and time period.

| Application periods | Groups | Parameter (Mean ± Standard Deviation) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Re-epithelialization | Granulation tissue | Angiogenesis | Dermal inflammation | Fibrosis | Type III collagen |
| **7 Days** | Control | 0.16 ± 0.4 | 1.0 ± 0.8 | 0.3 ± 0.5 | 2.2 ± 0.4 | 0.2 ± 0.4 | 2.3 ± 0.5 |
| | Vehicle | 0.16 ± 0.4 | 1.0 ± 0.8 | 0.3 ± 0.5 | 2.0 ± 0.6 | 0.3 ± 0.5 | 2.2 ± 0.4 |
| | Fito | 1.5 ± 0.5[b] | 0.7 ± 0.5 | 1.7 ± 0.5[c] | 0.3 ± 0.5[c] | 0.2 ± 0.4 | 1.5 ± 0.5 |
| | SK-0.5 | 2.0 ± 0.9[c] | 0.2 ± 0.4[a] | 2.0 ± 0.6[c] | 0.7 ± 0.5[c] | 0.2 ± 0.5 | 0.5 ± 0.5[c] |
| | SK-1 | 2.1 ± 0.7[c] | 0.2 ± 0.4[a] | 2.0 ± 0.9[c] | 0.5 ± 0.5[c] | 0.0 ± 0.0 | 0.5 ± 0.5[c] |
| | SE-0.5 | 1.5 ± 0.6[b] | 0.3 ± 0.5[a] | 2.2 ± 0.7[c] | 0.7 ± 0.5[c] | 0.3 ± 0.5 | 0.3 ± 0.5[c] |
| | SE-1 | 2.2 ± 0.9[c] | 0.3 ± 0.5[a] | 2.2 ± 0.8[c] | 0.2 ± 0.4[c] | 0.2 ± 0.0 | 0.2 ± 0.5[c] |
| **14 Days** | Control | 0.5 ± 0.54 | 0.7 ± 0.5 | 0.5 ± 0.6 | 1.8 ± 0.9 | 0.3 ± 0.5 | 1.8 ± 0.8 |
| | Vehicle | 0.5 ± 0.54 | 0.8 ± 0.9 | 0.5 ± 0.6 | 2.0 ± 0.6 | 0.5 ± 0.5 | 1.7 ± 0.5 |
| | Fito | 2.2 ± 0.4[c] | 0.0 ± 0.0[a] | 1.5 ± 0.5[b] | 0.2 ± 0.4[c] | 0.2 ± 0.4 | 0.5 ± 0.5[c] |
| | SK-0.5 | 2.2 ± 0.7[c] | 0.0 ± 0.0[a] | 2.5 ± 0.6[c] | 0.5 ± 0.5[c] | 0.0 ± 0.0 | 0.3 ± 0.5[c] |
| | SK-1 | 2.7 ± 0.5[c] | 0.0 ± 0.0[a] | 2.3 ± 0.5[c] | 0.5 ± 0.5[c] | 0.0 ± 0.0 | 0.0 ± 0.0[c] |
| | SE-0.5 | 2.2 ± 0.8[c] | 0.0 ± 0.0[a] | 2.7 ± 0.5[c] | 0.5 ± 0.4[c] | 0.0 ± 0.0 | 0.0 ± 0.5[c] |
| | SE-1 | 2.3 ± 0.5[c] | 0.0 ± 0.0[a] | 2.5 ± 0.6[c] | 0.2 ± 0.4[c] | 0.0 ± 0.0 | 0.0 ± 0.0[c] |

Measuring the average ± SD and Min-Max.

[a] ANOVA analysis (post-hoc: Tukey's HSD) was performed ($P < 0.05$).

[b] ANOVA analysis was performed ($P < 0.01$).

[c] ANOVA analysis was performed ($P < 0.001$). (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *Salvia kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *Salvia euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

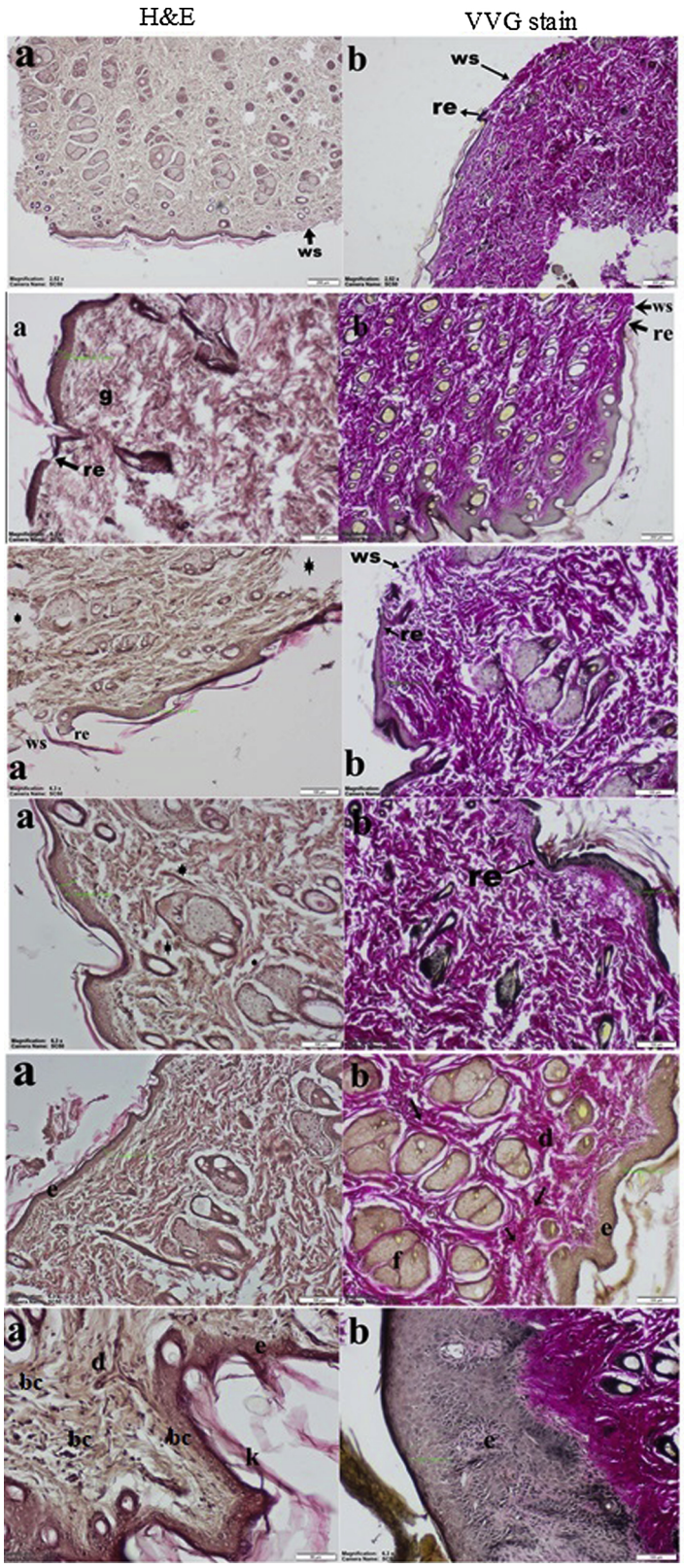


**Fig. 3.** Histological appearance of wound healing and epidermal/dermal re-modeling in control, vehicle and Fito groups on both 7 and 14 days. Control-7/Control group on day 7: (A) Re-epithelialization was limited, any alteration was not observed on wound surface. Necrotic was seen in dermis. (B) The wound was still not healed. Control-14/Control group on day 14: (A) Re-epithelialization was limited area, epidermis was not strictly completed, granulation tissue with thin collagen fibers was observed, necrotic areas in dermis was seen. (B) Epidermis was not still completely regenerated. Vahicle-7/Vehicle group on day 7: (A) Re-epithelialization was seen only limited area, wound surface could still be observed. Necrosis in dermis layers were indicated by asters. (B) Re-epithelialization was seen only limited area, wound surface could still be observed. Vehicle-14/Vehicle group on day 14: (A) Epidermis was observed as a very thin layer, and underlying dermis had very thin collagen fibers. Asters indicated necrotic area in dermis layer. (B) Epidermis was observed as a very thin layer. Fito-7/Fito group on day 7: (A) Granulation tissue with thin collagen fibers and more fibroblasts were seen in dermis layer. (B) Dermis, hair follicle and thick bundles of collagen fibers were observed. Fito-14/Fito group on day 14: (A) Epidermis and underlying dermis layers were seen. (re: re-epithelialization, g: granulation tissue, ws: wound surface, aster: necrotic area, e: epidermis, d: dermis, f: hair follicle, arrow: thick bundles of collagen fiber, k: keratinization, bc: blood capillary). (Fito: Fito® cream group).

tested groups on the 14th day indicated that granulation tissue thickness ($P < 0.05$) and type III collagen ($P < 0.001$) levels of Fito and SK and SE ointment (0.5% and 1% (w/w)) groups were significantly lower than control and vehicle. Angiogenesis in all treatment groups on the 7th day and 14th day were significantly increased when compared to control and vehicle groups. Angiogenesis values for SK and SE ointments ranging from 2.0 ± 0.6 to 2.7 ± 0.5 were found to be the most effective treatments at both 7 and 14 days, *i.e.*, even more efficient than Fito® cream at 1.7 ± 0.5 and 1.5 ± 0.5. Comparison of the test groups with control and vehicle showed that the test groups had significantly lower dermal inflammation levels on both 7 and 14 days ($P < 0.001$). When comparing the efficiency of Fito® cream and plant ointments,

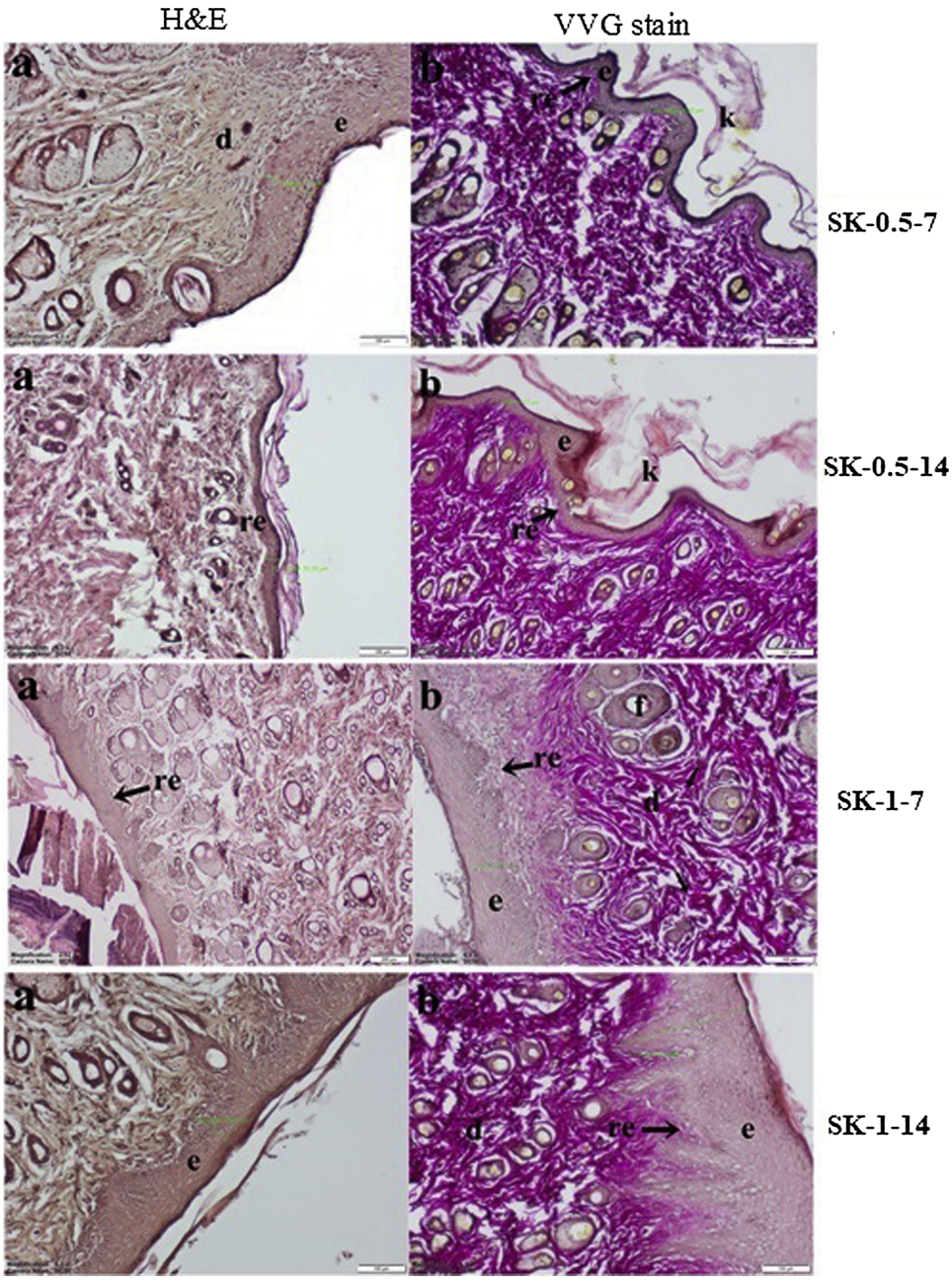


**Fig. 4.** Histological appearance of wound healing and epidermal/dermal re-modeling in SK-0.5 and SK-1 groups on both 7 and 14 days. SK-0.5-7/SK-0.5 group on day 7. (A) Epidermis and underlying dermis layer were seen. (B) VVG stain. SK-0.5-14/SK-0.5 group on day 14. (A) H&E stain. (B) VVG stain. SK-1-7/SK-1 group on day 7. (A) H&E stain. (B) Re-epithelialization and underlying granulation tissue were seen. Thick collagen fibers and hair follicles present in dermis layer. SK-1-14/SK-1 group on day 14. (A) H&E stain. (B) Epidermis was completely regenerated, and granulation tissue entirely removed. (e: epidermis, d: dermis, k: keratinization, re: re-epithelialization, f: hair follicle, arrow: thick collagen fiber). (SK-0.5: 0.5% (w/w) *S. kronenburgii* ointment group. SK-1: 1% (w/w) *S. kronenburgii* ointment group).

dermal inflammation values ranged from 0.2 ± 0.4 to 0.7 ± 0.5 for plant ointments which reduced dermal inflammation nearly as much as Fito® cream at 7 (0.3 ± 0.5) and 14 days (0.2 ± 0.4). SE-1 was found to be as effective as Fito® cream on both 7 and 14 days with a 0.2 ± 0.4 dermal inflammation value at both time points. No statistically significant differences were observed in fibrosis scores on days 7 and 14 (Table 6).

On day 7, granulation tissue with thin collagen fibers in the dermis, hair follicle, and thick collagen fiber bundles in the Fito group (Fig. 3); epidermis, underlying dermis layer, keratinization and re-epithelialization in SK-0.5 group; re-epithelialization, underlying granulation tissue, thick collagen fiber, and hair follicle in dermis layer, and epidermis in SK-1 group (Fig. 4); almost regenerated epidermis and re-epithelialization in SE-0.5 group and completely regenerated epidermis, re-epithelialization and keratin in SE-1 group (Fig. 5) were observed. On day 14, epidermis, underlying dermis layer, keratinization and blood capillary in Fito group (Fig. 3); re-epithelialization, keratinization, and epidermis in SK-0.5 group; completely regenerated epidermis, entirely removed granulation tissue, dermis and re-epithelialization in SK-1 group (Fig. 4); almost regenerated epidermis and re-epithelialization in SE-0.5 group, and completely regenerated epidermis and re-epithelialization in SE-1 group (Fig. 5) were observed.

### 3.5. Genotoxicologic study

The genotoxic potential of test materials and their probable oxidative damage on DNA were evaluated in peripheral blood samples using MD and comet assays (Table 7). The genotoxic study results indicated that after 7-day applications, treatment with SE ointments (0.5% and 1% (w/w)) led to reduce values of GDI and DCP ($P < 0.001$), and frequency of MN ($P < 0.01$) that were statistically significant when compared to control and Fito treatment. Additionally, GDI value for SK-0.5 and GDI and DCP values for vehicle were significantly lower than control and Fito ($P < 0.01$). The lowest GDI and DCP values, and MN frequency were observed in the 7 day SE-0.5 and SE-1 groups at 49.3 ± 3.63, 4.66 ± 1.40, and 1.33 ± 0.21; and 72.0 ± 6.72,

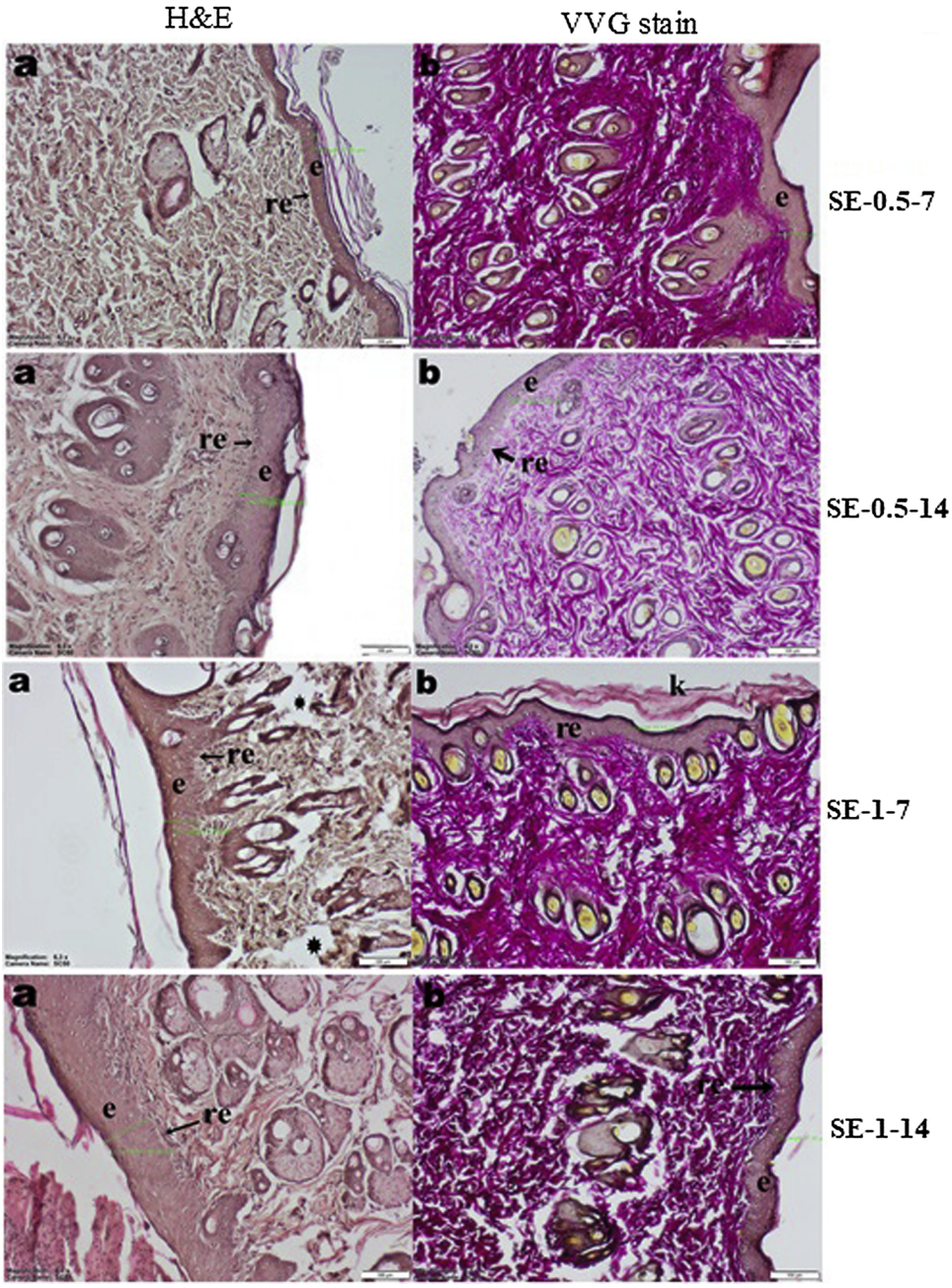


**Fig. 5.** Histological appearance of wound healing and epidermal/dermal re-modeling in SE-0.5 and SE-1 groups on both 7 and 14 days. SE-0.5-7/SE-0.5 group on day 7. (A) Epidermis was almost regenerated. (B) VVG stain. SE-0.5-14/SE-0.5 group on day 14. (A) Epidermis was almost regenerated. (B) VVG stain. SE-1-7/SE-1 group on day 7. (A) Epidermis was re-generated. (B) Epidermis was completely re-generated. SE-1-14/SE-1 group on day 14. Epidermis was completely regenerated (A) H& E stain. (B) VVG stain. (re: re-epithelialization, e: epidermis, aster: necrotic areas in dermis, k: keratin). (SE-0.5: 0.5% (w/w) *S. euphratica* var. *euphratica* ointment group. SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

**Table 7**
Micronucleus and comet assay results per treatment and length of application.

| Groups | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Vehicle | Fito | SK 0.5 | SK-1 | SE-0.5 | SE-1 | Positive control (MMC- 2 mg/kg) |
| **7-Day application** | | | | | | | | |
| GDI | 368.1 ± 7.04 | 146.66 ± 3.0[a] | 311.3 ± 20.39 | 195.8 ± 18.2[a] | 311.6 ± 12.6 | 49.3 ± 3.63[b] | 72.0 ± 6.72[b] | 237.00 ± 15.64 |
| DCP | 97.0 ± 1.52 | 39.3 ± 3.51[a] | 84.8 ± 5.07 | 60.6 ± 5.93 | 93.0 ± 2.11 | 4.66 ± 1.40[b] | 12.1 ± 2.50[b] | 84.83 ± 3.18 |
| MN/2000 | 3.66 ± 0.33 | 4.3 ± 0.57 | 3.50 ± 0.22 | 3.16 ± 0.30 | 3.16 ± 0.30 | 1.33 ± 0.21[a] | 1.50 ± 0.22[a] | 5.33 ± 0.33 |
| **14-Day application** | | | | | | | | |
| GDI | 345.8 ± 12.7 | 193.6 ± 3.51 | 85.6 ± 3.77[a] | 39.8 ± 1.72[b] | 85.6 ± 14.0[a] | 367.5 ± 0.88 | 368.1 ± 2.76 | 237.00 ± 15.64 |
| DCP | 95.8 ± 4.16 | 55.5 ± 1.52 | 10.8 ± 1.49[b] | 2.00 ± 0.57[b] | 17.3 ± 5.25[b] | 100.0 ± 0.00 | 100.0 ± 0.00 | 84.83 ± 3.18 |
| MN/2000 | 4.33 ± 0.42 | 5.0 ± 0.00 | 1.66 ± 0.21[a] | 1.50 ± 0.22[a] | 1.33 ± 0.21[a] | 4.00 ± 0.25 | 4.00 ± 0.25 | 5.33 ± 0.33 |

ANOVA analysis (post-hoc: Tukey's HSD) was performed. Measuring the average ± SD.

[a] $P < 0.01$.

[b] $P < 0.001$. GDI: Genetic Damage Index; DCP: Damaged Cell Percent; MMC: Mitomycin-C. (Control: Control group; Vehicle: Vehicle group; Fito: Fito® cream group; SK-0.5: 0.5% (w/w) *Salvia kronenburgii* ointment group; SK-1: 1% (w/w) *S. kronenburgii* ointment group; SE-0.5: 0.5% (w/w) *Salvia euphratica* var. *euphratica* ointment group; SE-1: 1% (w/w) *S. euphratica* var. *euphratica* ointment group).

12.1 ± 2.50, and 1.50 ± 0.22, respectively. Statistically significantly differences were not found between the SK-1, Fito, and control groups after 7 days of treatment.

After 14-day applications, treatment with SK ointments (0.5% and 1% (w/w)) and Fito® cream led to decrease GDI and DCP values, and MN frequency between the ranges of 85.6 ± 3.77–39.8 ± 1.72, 17.3 ± 5.25–2.00 ± 0.57, and 1.66 ± 0.21–1.33 ± 0.21, respectively. When compared to other tested groups statistically significant differences were determined in GDI ($P < 0.01$ and $P < 0.001$) and DCP ($P < 0.001$), and MN frequency ($P < 0.01$) for Fito, SK-0.5, and SK-1 groups. The significantly lowest GDI and DCP values were observed in the SK-0.5 group at 39.8 ± 1.72 and 2.00 ± 0.57 ($P < 0.001$), and for MN frequency in the SK-1 group at 1.33 ± 0.21 ($P < 0.01$). There were no statistically significant differences between the SE ointment (0.5% and 1% (w/w)), control, and vehicle groups after 14 days of treatment.

### 3.6. Antimicrobial study

The antimicrobial study results indicated that both plants inhibited growth of all tested microbial strains. When compared to the reference antimicrobial agents Ampicillin, Isoniazid, Ethambutol, and Fluconazole, the ethanol extracts obtained from aerial parts of SK and SE showed antimicrobial activity against six bacterial and three fungal strains with MIC values ranging between 250–31.25 µg/mL and 125-0.24 µg/mL, respectively. Both tested extracts had greater antibacterial activity against the gram-negative bacterial strain *A. baumannii* with a 62.5 µg/mL MIC value compared to reference drug Ampicillin with a 125 µg/mL MIC value. Against *M. tuberculosis* H37Rv, the ethanol extract of SE with a 0.24 µg/mL MIC value was found to be more effective than reference drugs Isoniazid and Ethambutol (0.97 and 1.95 µg/mL MIC values, respectively). The antifungal activity results showed that both tested extracts exhibited lower antifungal activity ranging from 62.5 to 125 µg/mL MIC values against the three tested fungal strains (*C. glabrata, C. tropicalis* and *C. parapsilosis*) when compared to the reference antifungal drug Fluconazole with 3.90, 15.62, and 3.90 µg/mL MIC values, respectively (Table 8).

### 3.7. Total phenolic, flavonoid contents and DPPH radical scavenging activity

The results of total phenolic and total flavonoid contents are detailed in Table 9. TPC and TFC of the SK were measured as 41.81 ± 1.858 µg GAE/mg and 33.62 ± 0.884 µg QE/mg, respectively. TPC and TFC of the SE were measured as 76.21 ± 1.815 µg GAE/mg and 43.43 ± 0.456 µg QE/mg, respectively.

DPPH radical scavenging activities of ethanol extracts at various concentrations (10–250 µg/mL) are shown in Fig. 6. Increased DPPH radical scavenging activities were observed at high concentrations. Positive control BHA was examined at 2.5–250 µg/mL concentrations. The ethanol extracts of both SK and SE showed significant inhibition levels at 250 µg/mL concentration with the values of 72.17 ± 0.84% and 87.08 ± 1.80%, respectively.

**Table 9**
Total phenolic and flavonoid contents of the ethanol extracts of the studied plants.

| Sample | TPC (µg GAE/mg extract) | TFC (µg QE/mg extract) |
|---|---|---|
| SK | 41.81 ± 1.858 | 33.62 ± 0.884 |
| SE | 76.21 ± 1.815 | 43.43 ± 0.456 |

Values are the mean ± SD of the data (n = 3). TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent, QE: Quercetin equivalent, SK: *Salvia kronenburgii*, SE: *Salvia euphratica* var. *euphratica*.

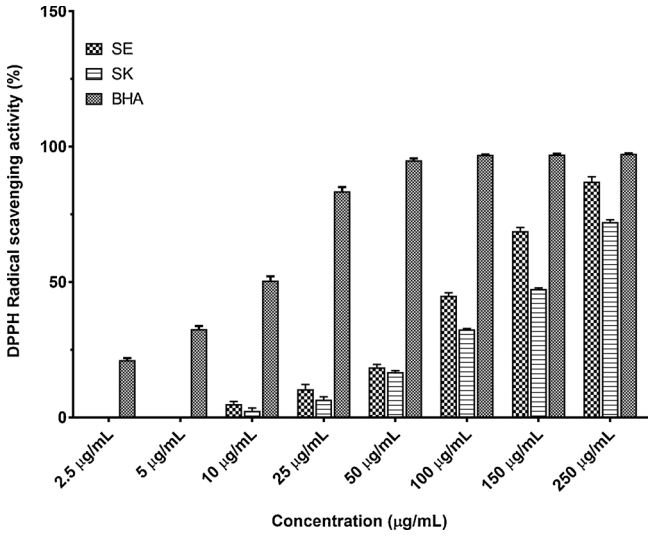


**Fig. 6.** Radical scavenging activities of ethanol extracts of SK and SE, and standard antioxidant BHA by DPPH assay. Values are the mean ± SD of the data (n = 3). (SK: *Salvia kronenburgii*, SE: *Salvia euphratica* var. *euphratica*, BHA: Butylated hydroxyanisol).

## 4. Discussion

After a wound occurs, the repair procedure begins as a normal biological phenomenon [46]. Wound healing is divided into four highly programmed phases including hemostasis, inflammation, proliferation (contraction, granulation, epithelialization, and tissue formation), and remodeling. Wound healing processes require coordination of

**Table 8**
Minimum inhibitory concentration values for tested plant extracts and reference drugs against bacterial and fungal strains (µg/mL).

| Tested microorganisms | Code | *Salvia kronenburgii* | *Salvia euphratica* var. *euphratica* | Reference Drugs | | | |
|---|---|---|---|---|---|---|---|
| | | | | Ampicillin | Isoniazid | Ethambutol | Fluconazole |
| **Bacterial strains** | | | | | | | |
| *Staphylococcus aureus* | ATCC 25925 | 125 | 125 | 31.25 | NT | NT | NT |
| *Bacillus subtilis* | ATCC 6633 | 125 | 125 | 0.9 | NT | NT | NT |
| *Escherichia coli* | ATCC 25923 | 250 | 125 | 15.62 | NT | NT | NT |
| *Acinetobacter baumannii* | ATCC 02026 | 62.5 | 62.5 | 125 | NT | NT | NT |
| *Aeromonas hydrophila* | ATCC 95080 | 125 | 125 | 31.25 | NT | NT | NT |
| *Mycobacterium tuberculosis* | H37Rv | 31.25 | 0.24 | NT | 0.97 | 1.95 | NT |
| **Fungal strains** | | | | | | | |
| *Candida glabrata* | ATCC 90030 | 62.5 | 62.5 | NT | NT | NT | 3.90 |
| *Candida tropicalis* | ATCC 750 | 62.5 | 125 | NT | NT | NT | 15.62 |
| *Candida parapsilosis* | ATCC 22019 | 62.5 | 62.5 | NT | NT | NT | 3.90 |

Values determined in duplicate with deviations within one two-fold dilution. NT: Not tested.

overlapping distinct cellular activities, involving phagocytosis, chemotaxis, mitogenesis, angiogenesis, synthesis of collagen and other matrix constituents, and interaction of extracellular matrix molecules, soluble mediators, and various cells that cooperate in the repair procedure [4]. For successful wound healing, all steps must occur in a suitable sequence and time frame [46,47]. This multi-factorial process of wound healing can fail and/or be delayed by infections caused by microorganisms, adverse effects like the presence of free radicals [46], and/or some metabolic diseases like DM [3].

Wound contraction, wound closure, and functional barrier re-organization are important markers in the healing process [5]. The type of damage plays a significant role in wound healing; therefore, healing stages of wound models such as incision, excision, burn, and dead-space are affected differently during the process [48]. These differences explain why the contraction ratios of test groups are different for excision and incision wound models on day 7. When compared to control and vehicle, SK and SE showed significant wound closure ratios with a range of 96.9%–99.9% in excision ($P < 0.05$) and incision ($P < 0.01$) wound models on day 14. According to contraction ratios on days 7 and 14, both concentrations of SK and SE ointments were found to be as effective as the reference drug, Fito® cream.

Skin biomechanical parameters are used for measurement of skin quality in the wounded area; therefore, these parameters are an important reflection of the subdermal organization of newly synthesized collagen fibrils in deposited collagen [47]. According to the biomechanical study results, 0.5% (w/w) ointments of both tested plants resulted in better skin quality than 1% (w/w) ointments with higher values for energy absorpsion capacity, maximum load, elasticity, toughness, maximum stress, and lower values for maximum deformation on day 7. SK-0.5 resulted in better skin quality than SK-1 with higher values of energy absorpsion capacity, maximum load, elasticity, and maximum stress whereas SE-1 resulted in better skin quality than SE-0.5 with higher values of energy absorpsion capacity, toughness, and maximum strain and lower value of stiffness on day 14. Furthermore, SK appeared to be more effective than SE at both 7 and 14 days and SK was also found to be nearly as effective as the reference drug Fito® cream according to 14 days results.

An increase in hydroxyproline levels in healing tissues is evidence of rapid wound healing and measurement of hydroxyproline is an acceptable method for predicting the amount of collagen synthesis [49]. After 7 days applications, Fito® cream, SK and SE ointments (0.5% and 1% (w/w)) contributed to regulation of collagen fibrils and collagen formation by increasing the hydroxyproline levels; however, after 14 days of application, decreasing hydroxyproline levels were observed in the same groups, and these results paralleled histopathology results. Due to increasing reactive oxygen species production and/or decreasing antioxidant scavenging activities, increased MDA (lipid peroxide formation) levels were found in wound tissues of diabetic rats [50]. In our study, decreased MDA levels were observed in Fito® cream, SK and SE ointment-treated groups on both 7 and 14 days compared to control groups. Also, MDA levels of SK and SE ointment-treated groups were lower than in the Fito® cream-treated group on day 7. The wound healing process is associated with antioxidant activity, because of increasing colloid synthesis and removing free oxygen radicals, antioxidants significantly accelerate wound healing [51]. Recently, the contribution of antioxidants on excisional wounds of normal and STZ-induced diabetic rats have been investigated and decreased GSH levels were found in 7-day wound tissues of diabetic rats. Due to increasing oxidative stress, serum levels of the antioxidants decreased in diabetic animals. GSH which is known as a non-enzymatic antioxidant, regulates the early phases of wound healing; therefore, lack of GSH may play a role in delaying the wound healing process in diabetic rats [52]. In the present study, increasing GSH levels were observed in the SE-0.5 group on day 7 and in the Fito® cream, SK-0.5, SK-1, and SE-0.5 groups on day 14. Evidence from human and animal studies indicate that NOx plays a key role in the wound repair process due to its beneficial effects on inflammation, angiogenesis, matrix deposition, cell proliferation, and remodeling. There are strong correlations between the synthesis of NOx, and collagen deposition and increased mechanical strength. In the early stages of wound healing (1–5 days), increasing NOx levels (nitrite [$NO_2$] and nitrate [$NO_3$]) in the fluid of subcutaneous wounds and increasing urinary nitrate excretion in excisional wounds were found, after which NOx levels decrease. Deficiency of NOx is associated with impaired wound healing and decreasing collagen deposition and breaking strength in incisional wounds. Correlations have been found between impaired wound healing and decreased cutaneous NOx levels in diabetic wounds. Also, significantly decreased NOx levels were found in STZ-induced diabetic animals [53]. When the 7 and 14 days results of the present study were compared, except for in the SK-0.5 group, NOx levels of all test groups were higher on day 7 than on day 14. After 14 days, significantly reduced NOx levels were observed in the SK-1, SE-0.5, and SK-1 groups and these levels were found to be significantly lower than in the Fito® cream group.

For histopathological evaluation, re-epithelialization, thickness of granulation tissue, angiogenesis, extent of dermal inflammation, presence of fibrosis, and type III collagen deposition were studied. Re-epithelialization, which is used as an important parameter of successful wound closure, is one of the crucial steps of wound healing and is characterized by migration of keratinocytes and increased proliferation over the wounded area. In all different types of chronic wounds, the epithelialization process is impaired and the absence of re-epithelialization results in impaired wounds [54]. In the present study, increased re-epithelialization levels in Fito, SK, and SE groups were observed on both 7 and 14 days. Keratinocytes are important in maintaining the epidermis and restoring it after wounding. As a successful response to epidermal injury, the hair follicles also join in re-epithelialization of wound defects [54]. Collagen plays an important role in wound contraction and skin quality depends on increasing collagen content [14]. Formation of collagen fibrils is also important for wound healing [55]. During wound healing, type III collagen, which is known as the major constituent of the granulation tissue, is replaced by type I collagen which is the main structural constituent of the dermis [56]. This data supports the reduced granulation tissue thickness and type III collagen amount in the Fito, SK, and SE groups on day 14. The healing properties of phytomedicine are generally related to angiogenesis which is another important step of wound healing [4]. Angiogenesis, which is classified as new blood capillary formation of pre-existing capillaries, plays an important role in physiological processes like growth, repair, and/or some pathological conditions [4,57]. To supply nutrients for maintenance of cell metabolism, creating an intact delivery system, and removing unwanted formations, well-organized angiogenesis is essential during the wound repair process [4]. In our study, increasing angiogenesis levels in Fito, SK, and SE groups contributed to the formation of new blood capillaries on both 7 and 14 days. During the healing process the inflammatory response is important to contribute resistance against microbial contamination, whereas long term inflammation leads to a delay in the healing phases [15]. In the present study, decreased dermal inflammation levels were observed in Fito® cream-, SK- and SE-treated groups on both 7 and 14 days.

The genotoxic potential of STZ has been studied in rat peripheral blood and bone marrow previously. The induction of MN was observed in peripheral blood and bone marrow of neonatal and young rats, and in bone marrow of adult rats. Furthermore, the genotoxic potential of STZ at 30 mg/kg concentration (for 3 days) was tested using the comet assay in bone marrow and peripheral blood of young rats, and results indicated that STZ induced significant DNA damage [58]. In the present study, we evaluated the genotoxic effects of materials in peripheral blood samples of STZ-induced diabetic rats. Compared to control, decreasing GDI and DCP values and MN frequency were observed in Fito, SK, and SE groups. Genotoxic study results indicated that decreasing GDI and DCP values, and MN frequency were dependent on application periods (7 and 14 days), treatment dose (0.5% and 1% (w/w)), and

tested plants (SK and SE). According to our results, after the 7-day treatment, GDI and DCP values, and MN frequency indicated that SE-0.5 and SE-1 were more effective than the reference drug Fito® cream and SK-0.5 and SK-1. Moreover, SE-0.5 was more effective than SE-1. Despite these results, after 14-day treatments, efficiency of SE ointments was reduced according to increasing GDI and DCP values, and MN frequency. Our results indicated that short term usage (7 days) of SE ointments (0.5% and 1% (w/w)) were more effective in reducing oxidative damage to DNA than long term usage (14 days). Evaluation of treatments for 7 days showed that GDI and DCP values and MN frequency of SK ointments (0.5% and 1% (w/w)) were not as effective as Fito® cream, except for the GDI value for SK-0.5. After 14 days of SK-0.5 and SK-1, GDI and DCP values, and MN frequency were reduced. These results were comparable to Fito® cream results. Additionally, SK-0.5 was more effective than Fito® cream. Our results showed that long term usage (14 days) of SK ointments more efficiently reduced oxidative damage to DNA than short term usage (7 days).

The macroscopic, biomechanical, biochemical, and histopathological evidence indicate that both tested plants were as effective as the reference drug Fito® on incisional and excisional wound healing in STZ-induced diabetic rats for two application periods (7 and 14 days). Additionally, SK was more effective than SE at both 7 and 14 days. Both concentrations of SK (0.5% and 1% (w/w)) showed significant wound healing potential, but according to genotoxic results the 0.5% (w/w) concentration reduced DNA damage more than the 1% (w/w) concentration. The 0.5% (w/w) concentration of SE showed better wound healing potential than the 1% (w/w) concentration on day 7 while the 1% (w/w) concentration of SE was more effective than the 0.5% (w/w) concentration on day 14. However, genotoxic results indicated that using SE for 7 days reduced DNA damage more than using SE for 14 days. According to the genotoxic results and histopathological evidence, SE-0.5 almost regenerated the epidermis and SE-1 completely regenerated the epidermis on day 7; however, usage of SE ointments may be limited to 7 days to reduce oxidative damage to DNA.

Generally, gram-negative bacteria are much more resistant than gram-positive bacteria [59] and the cell wall structure of gram-negative bacteria is more complex than that of gram-positive bacteria. Because of the different arrangement of outer membranes in gram-negative and gram-positive bacteria, penetration of macromolecules vary during treatment [13] and also against natural components with gram-negative bacteria being more resistant than gram-positive bacteria. Additionally, gram-negative bacteria's hydrophilic cell wall structure, which contains lipopolysaccharides, inhibits accumulation of hydrophobic oils, extracts, and steroids, and penetration of these substances through the target cell membrane. The literature data confirms why gram-positive bacteria exhibit greater sensitivity against natural products than gram-negative bacteria [59]. In the present study, ethanol extracts of two tested plants had antimicrobial properties against all tested microorganisms including two gram-positive and three gram-negative bacteria, *M. tuberculosis,* and three fungi. Both tested plants showed stronger antibacterial activity against the gram-negative nosocomial pathogen *A. baumannii* which is a serious public healthcare problem worldwide due to its ability to gain resistance to all classes of antimicrobial agents used against it [60]. Recently, *A. baumannii* has received clinically attention in intensive care units and is known to be responsible for various infections including surgical wound infections, bacteremia, ventilator-associated pneumonia, meningitis, urinary tract, and bloodstream infections [61]. In the present study, the ethanol extract of SE showed stronger antimycobacterial activity against *M. tuberculosis* which has the ability to gain resistance to the two most used antimycobacterial agents (Isoniazid and Rifampicin) in the treatment of tuberculosis [39]. This result also supports traditional usage of the *Salvia* species as a tea for alleviating night sweats of patients suffering from tuberculosis [10]. Antibacterial and antifungal activities of several *Salvia* species were reported against various microorganisms [10,13,62]. Literature data supports our findings and according to our results, SK and SE can be promising sources of antimicrobial agents for the treatment of infections caused by both microorganisms *M. tuberculosis* and *A. baumannii*. Moreover, microbial infections caused by various microorganisms including *Staphylococcus*, *Pseudomonas*, *e.g.* were demonstrated in chronic wounds. Additionally, investigation on wound infections in animal models indicated that epithelialization was significantly inhibited by polymicrobial infection with *S. aureus* and *Pseudomonas aeruginosa* more than infection with a single species [54]. In the present study, ethanol extracts of SK and SE showed antimicrobial activity against *S. aureus* and other tested microorganisms with different MIC values; therefore, we propose that both tested plants contribute to wound healing due to their antimicrobial properties.

In this study we used ethanol which is used on a broad range of polar constituents [63] such as phenolics and terpenoids [64] as an extraction solvent. *Salvia* species are a rich source of polyphenols including phenolic acids and flavonoids [39]. Flavonoids (quercetin, apigenin and luteolin) and phenolic acids (gallic, rosmarinic, chlorogenic, and caffeic acids) [65] were reported in *Salvia* species and most *Salvia* species contain caffeic acid derivatives as the major phenolic acids [15]. Presence of phenolic acids (rosmarinic [major component], gallic, chlorogenic, and caffeic acids) [66], diterpenoids (horminone and 7-O-acetylhorminone) [11], and volatile organic compounds (*β*-caryophyllene [9], 1,8-cineole, limonene, carvone, *trans*-linalool oxide, *cis*-linalool oxide [6]) from aerial parts of SK and the presence of phenolic acids including rosmarinic (major component), caffeic, protocatechuic, gallic, chlorogenic, p-coumaric, o-coumaric, and ferulic acids and trans-pinocarvyl acetate and eucalyptol (major components of essential oil) from aerial parts of SE were determined previously [67]. Antioxidant, antimicrobial, and wound healing activities of phenolics [15,16] and antimicrobial properties of phenolic acids, flavonoids, essential oils, terpenoids *e.g.* have also been reported [64]. Studies on di- and tri-terpenoids of *Salvia* species which are growing in Turkey demonstrated that triterpenoids showed almost no antimicrobial activity against a yeast, *Candida albicans* and standard bacteria [10] while phenolic constituents of the *Salvia* species were associated with antimicrobial properties of the genus [68]. *In vitro* antioxidant activity of methanol and dichloromethane extracts of aerial parts from SE was studied using DPPH free radical-scavenging and β-carotene/linoleic acid systems and results showed that the plant had strong antioxidant activity [67]. Potent antioxidative effects of phenolic compounds obtained from various *Salvia* species have been reported [65]. According to the literature, phenolic compounds well-known as potent antioxidants, contribute to the healing process and phenolic compounds such as flavonoids accelerate wound healing [15]. Ozay et al. [5] demonstrated the wound healing effects of luteolin on excision and incision wound models in non-diabetic and STZ-induced diabetic rats. Also, flavonoids enhanced collagen fibril formation in wound tissues [5] and they are responsible for activation of the anti-inflammatory system that acts against lipid peroxidation [16]. Anti-inflammatory and wound healing activities of caffeic and rosmarinic acids have been shown previously [15]. Moreover, rosmarinic acid had inhibitory effect on 5-lipoxygenase and TNF-α release. Besides phenolics, anti-inflammatory properties of triterpenoids (oleanolic and ursolic acids) and diterpenoid (carnosol) of *Salvia officinalis* L. have been reported [57]. In the present study, total phenolic and flavonoid contents and DPPH radical scavenging activities of the ethanol extracts obtained from aerial parts of SK and SE were evaluated and results indicated that there was a positive correlation between the TPC, TFC and antioxidant capacity of the tested extracts. In addition to their antimicrobial properties, we propose that both plants contribute to wound healing due to their antioxidant properties.

There are a few studies on the wound healing effects of *Salvia* species [14–16]; however, there is no study on their wound healing effects in diabetic rats. Karimzadeh and Farahpour [16] studied *in vivo* wound healing activity of hydroethanolic extract obtained from the leaf of *S. officinalis*. After treatment with three concentrations (1%, 3%, and

5%) of plant extract on excision and incision wounds, they found that all concentrations had wound healing potential when compared to control. Among them the 5% concentration was found to be the most effective dose with increased wound contraction percentage, re-epithelialization period, breaking strength ratio and up-regulated content of hydroxyproline. Moreover, the plant accelerated formation of new vessels and distribution of fibroblasts. Salimikia et al. [69] studied wound healing effect of methanolic extract obtained from the aerial parts of *Salvia multicaulis* Vahl. on excision wounded rats for 14 days. They tested three concentrations (5%, 10%, and 30%) of the extract and found that all concentrations had wound healing potential when compared to control. Especially, the 5% concentration was found to be the most effective one with 99.38% contraction rate and the highest fibril levels. Narayan et al. [14] investigated the wound healing potential of *Salvia splendens* Sellow ex Roemer & J. A. Schultes on excision and incision wound models in albino mice. Different concentrations (5%, 10%, and 15% (w/w)) of methanol extract obtained from the leaves were tested during 20 days for excision wounds and during 10 days for incision wounds with Nitrofurazone ointment (0.2% w/w) used as a reference drug. According to various biochemical parameters (collagen, mucopolysaccharides, DNA and protein contents of granulation tissues) and percentage of wound contraction, the ointment including 15% (w/w) extract showed 100% healing at the end of the 18$^{th}$ day and was found to be as effective as the reference drug Nitrofurazone. Suntar et al. [15] investigated the *in vivo* wound healing effects of ethanol extracts obtained from aerial parts of *Salvia cyanescens* Boiss and Bal. and *Salvia cryptantha* Montbret and Bentham on excision and incision wound models. They tested their compounds on excision wounds in mice for 12 days and incision wounds in rats for 10 days and used Madecassol® as a reference drug. They found that extracts of *S. cryptantha* and *S. cyanescens* exhibited 56.5% and 34.6% contractions, respectively, while Madecassol® showed 100% contraction. Wound tensile strength values of *S. cryptantha* and *S. cyanescens* and Madecassol® were 33.2%, 22.5%, and 51.8%, respectively. According to their results both tested plant extracts were not found as effective as the reference drug [15]. Angiogenic activity of *Salvia miltiorrhiza* Bunge has been reported and significant stimulation of proliferation and migration of human umbilical vein endothelial cells (HUVECs) were observed which was responsible for activation of different signaling pathways [4]. Chen et al. [70] demonstrated that *S. miltiorrhiza* achieved enhanced proliferation of Detroit 551 fibroblast cells and the plant had the ability to increase the collagen production in the same cell line. Moreover, crude extract of S. *miltiorrhiza* and salvianolic acid B isolated from the same plant showed increased expression of matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor type-2 (VEGFR-2), and Tie-1 receptor [4].

## 5. Conclusion

Ideal wound healing is characterized by successful closure in a very short time without any side effects [47]. Despite the negative effects of DM, in our study the wound healing phenomenon was well orchestrated in diabetic rats and topical applications of the tested plants showed strong wound healing effects with significant antimicrobial and antioxidant activities. This is the first report describing wound healing, antimicrobial and antioxidant activities of both SK and SE. Moreover, healing effects of *Salvia* species on diabetic wounds were studied for the first time. As an alternative to synthetic drugs with expensive costs and adverse effects, our results indicate that two endemic Turkish *Salvia* abundantly present in nature, appear to be promising wound healing agents for the treatment of non-healing and persistent diabetic wounds and promising antimicrobial agents for treatment of infectious diseases caused by *M. tuberculosis* and *A. baumannii* with their antioxidant potential.

## Authors' contributions

S.G.: Extraction, Total phenolic content, Total flavonoid content, DPPH assay, preparing ointments, observing animals, induction of diabetes with STZ, creating wounds, topically ointment application, obtaining wounded tissues from animals, writing the manuscript. Y.Ö.: Planning of experimental studies, obtaining wounded tissues from animals, collecting experimental data, creating wounds, surgical applications, writing the manuscript. M.K.: Histopathologic studies, evaluation of the histopathological data, statistical analyses of all data. C.U.: Performing biomechanical studies, observing animals, induction of diabetes with STZ, topically ointment application, photographing tissues for macroscopic studies. *E.G.*Ö.: Histopathologic studies. Z.Y.: Biochemical studies. M.Ü.: Antimicrobial activity studies. G.G.: Commet assay. A.Ç.: Micronucleus assay. Y.Ç.: Planning of experimental studies, observing animals, induction of diabetes with STZ, creating wounds, obtaining wounded tissues from animals. A.K.: Collecting plant materials. All authors read and approved the final manuscript.

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## Conflict of interest

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