Wound healing activity of *Salvia huberi* ethanolic extract in streptozocin-induced diabetic rats

Objective: The aim of this study was to examine the in vivo wound healing potential of *Salvia huberi* Hedge (endemic to Turkey) on excision and incision wound models in diabetic rats.
Method: Male Wistar albino rats, 3–4 months old and weighing 180–240g were used. The animals were randomly divided into five groups including Control, Vehicle and Fito reference, and two different concentrations (0.5% and 1% weight/weight (w/w)) of ethanol extract of *Salvia huberi* were investigated in both wound models on streptozocin-induced diabetic rats using macroscopic, biomechanical, biochemical, histopathological, genotoxic and gene expression methods over both seven and 14 days. Fito cream (Tripharma Drug Industry and Trade Inc., Turkey) was used as the reference drug.
Results: A total of 60 rats were used in this study. *Salvia huberi* ointments at 0.5% and 1% (w/w) concentrations and Fito cream showed 99.3%, 99.4% and 99.1% contraction for excision wounds, and 99.9%, 97.0% and 99% contraction for incision wounds, respectively. In *Salvia huberi* ointments and Fito cream groups, re-epithelialisation increased dramatically by both day 7 and day 14 ($p<0.05$). By day 14, low hydroxyproline and malondialdehyde (MDA) levels, and high glutathione (GSH) levels were observed in the *Salvia huberi* ointment groups. After two application periods, damaged cell percent and genetic damage index values and micronucleus frequency of *Salvia huberi* ointment treatment groups were lower than Control and Vehicle groups ($p<0.001$). A growth factor expression reached a high level by day 7 in the Control group; in *Salvia huberi*-treated groups it was decreased.
Conclusion: The study showed that application of *Salvia huberi* ointments ameliorated the healing process in diabetic rats with excisional and incisional wounds and may serve as a potent healing agent.
Declaration of interest: The authors have no conflicts of interest.

diabetic rat • ethanolic extract • Salvia huberi • streptozocin • wound • wound care • wound dressing • wound healing

A wound is defined as a physical or thermal injury resulting in breakage or opening of the skin surface that disrupts normal skin anatomy and function.[1] Wound healing is an active process supported by multicellular activities. This process includes haemostasis, inflammation, proliferation and remodelling phases.[2] The repair process begins as a normal biological phenomenon after wounds occur, and all stages must proceed in an appropriate sequence and timeframe for ideal wound healing.[3] However, various factors may disrupt the wound healing phases, such as insufficient blood flow, impaired immunity and angiogenesis,[4] and deficiency in the production of growth factors and accumulation of collagen.[5] When the healing phases are disrupted, wounds become hard-to-heal and these wounds are mostly associated with diabetes, atherosclerosis, vasculitis and trauma.[6] Diabetes, the prevalence of which has been rising more rapidly in low- and middle-income countries than in high-income countries,[7] is one of the primary causes of impaired skin wound healing.[8] Diabetes is a chronic metabolic disorder with an additional increased oxidative stress condition. Excessive free radical production in diabetes interacts with lipids, proteins and nucleic acids, leading to the loss of membrane integrity, genetic mutations, and structural or functional changes in proteins.[9] Several medicinal plants are documented for the management of diabetes and considered to be valuable sources for potential new drugs.[9] one of them is *Salvia* L. (sage) that exhibits blood glucose reducing and antidiabetic features.[10]

Salvia is the most species-rich genus of Lamiaceae (the mint family), with 1000 species, and distributed extensively in tropical and temperate areas of the New and Old Worlds.[11] One of the main diversity centres of

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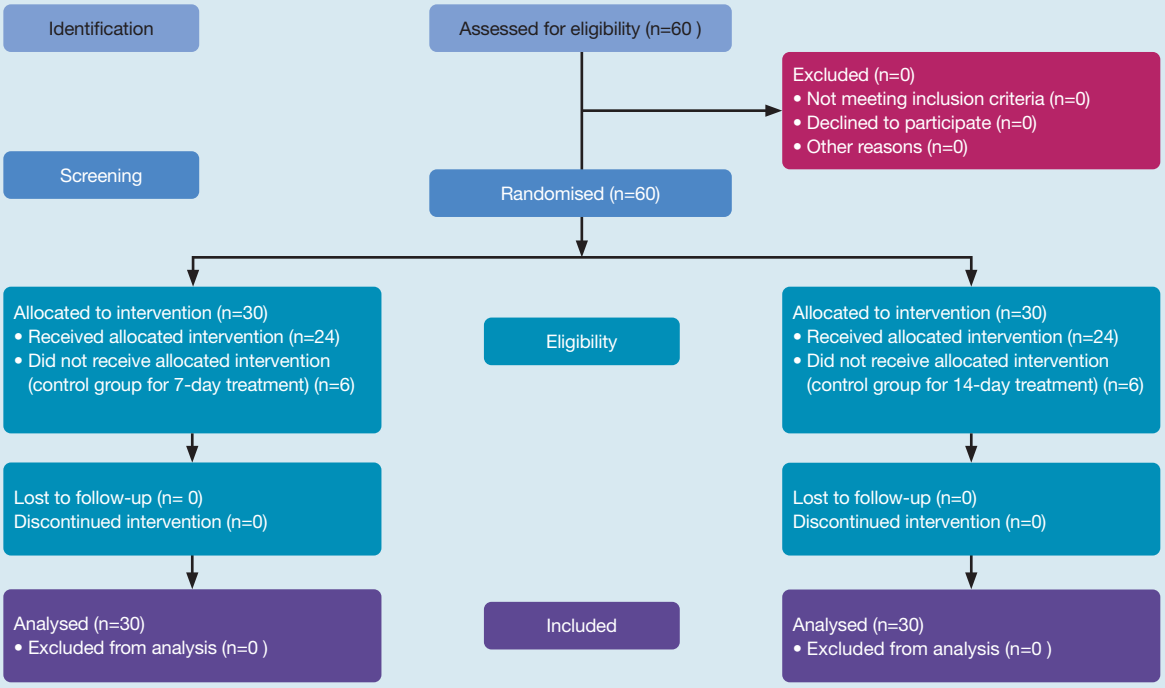
Fig 1. CONSORT flow diagram



the genus is Southwest Asia, where Turkey has the largest number of species (around 100, including 53 which are endemic).[12] Since ancient times, species of the genus have been widely used as a folk medicine for the treatment of the common cold, bronchitis, tuberculosis, wounds, malaria, microbial infections, inflammation, cancer, loss of memory, haemorrhage and hepatitis.[13] Through the combination of various extraction methods performed on the different parts of the plant (aerial, root, flower, seed etc.), the presence of flavonoids,[14,15] phenolic acids,[14] terpenoids,[15] essential oils,[16] fatty acids, tocopherols, amino acids and minerals[12,17] have all been reported. Moreover, antibacterial, antifungal,[13,18] wound healing,[19–21] antiproliferative,[22,23] cytotoxic[19,24] and antioxidant[19,25] activities of *Salvia* species have been indicated. *Salvia huberi* Hedge is an endemic species growing in Turkey.[11] According to the literature, there is no study on the wound healing effect of *Salvia huberi*. Therefore, in this study, we aimed to examine the in vivo wound healing potential of *Salvia huberi* on excisional and incisional wound models in diabetic rats.

Methods

Plant material

Salvia huberi was collected from Erzurum, Turkey and identified/confirmed by Dr Ahmet Kahraman (Department of Biology, Faculty of Arts and Science, Uşak University, Uşak, Turkey). The dried voucher specimen was stored in the Plant Systematics and Phylogenetics Research Laboratory, Uşak University (Voucher reference: A. Kahraman 2100).

Extraction

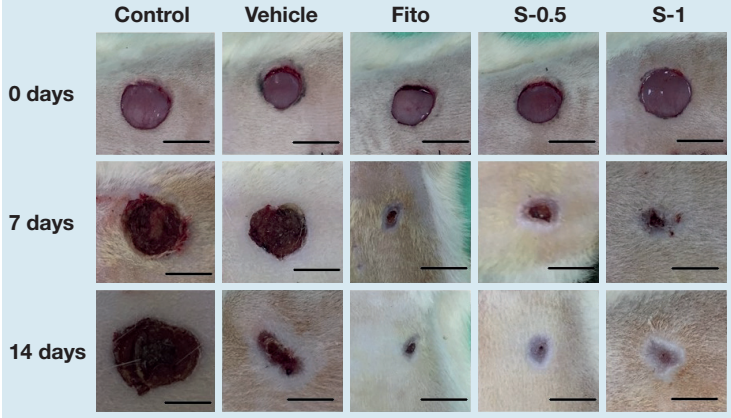
Aerial parts of the plant sample were air-dried and powdered then macerated (×3) with ethanol (96%, EtOH, Merck, Germany) (20ml of EtOH per 1g of plant) at room temperature. After filtering using filter paper

Table 1. Experimental design for diabetic rat wound treatment groups (n=60)

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
S-0.5	0.5% (w/w) *Salvia huberi* ointment	0.5% (w/w) *Salvia huberi* ointment	12
S-1	1% (w/w) *Salvia huberi* ointment	1% (w/w) *Salvia huberi* ointment	12

*For each group, n=6 for 7-day applications and n=6 for 14-day applications; w/w—weight/weight

Fig 2. Macroscopic view of excision wounds at 0, 7 and 14 days. Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight (w/w)) *Salvia huberi* ointment group; S-1—1% (w/w) *Salvia huberi* ointment group. Scale bar: 1.3cm



(Whatman Grade No.1; Sigma-Aldrich, Germany), solvent was evaporated at 35–40°C using a vacuum evaporator (Heidolph-Rotar TLR 1000; Heidolph Instruments, Germany) and the obtained plant extract was stored in the dark at 4°C until further use (extract yield: 6.18%, weight/weight (w/w)).

Ointment process

Propylene glycol:glycol stearate:liquid paraffin (6:3:1) mixture was used as a simple ointment base.[26] The simple ointment base was topically used for the treatment of the Vehicle group. As a reference drug, Fito cream (Tripharma Drug Industry and Trade Inc., Turkey) containing *Triticum vulgare* L. aqueous extract (15% (w/w); Tripharma Drug Industry, Turkey) was used. A suitable amount of extract was added to the simple ointment base for preparing 1% and 0.5% (w/w) plant ointments (S-1 and S-0.5, respectively). Depending upon group assignment to seven or 14 days, wounded areas were treated topically once daily with the ointments or reference drug (0.5g).

Animals and experimental protocol

A total of 60, healthy male Wistar albino rats, 3–4 months old and weighing between 180–240g, were used for the experiments. The animals, purchased from the Research Center of Experimental Animals (Mersin University, Mersin, Turkey), were individually kept in cages under standard conditions (humidity: 50–70%, light/dark cycle: 12/12 hours, temperature: 25±3°C) and fed a standard pellet diet and water ad libitum during the experiments (for seven and 14 days). The experimental protocol was approved by the Ethics Committee of Mersin University School of Medicine (Reg. No. 2016/05). All procedures were approved by the Animal Care and Use Committee at Mersin University and performed in accordance with institutional guidelines. The animals were randomly divided into five equal groups of 12 animals, with six animals from each group treated for seven days and six for 14 days. The experimental design of the study is presented in Fig 1 and Table 1.

Induction of diabetes

Streptozocin (STZ) (Sigma Chemical Co., US) freshly prepared in saline was administered to the animals via intraperitoneal injection (45mg/kg body weight). Then, 72 hours after STZ administration, a rapid glucometer (Bayer, Germany) was used to measure fasting blood glucose levels, and levels above 300mg/dl were accepted as diabetic.[19] All animals were hyperglycaemic throughout the experiments.

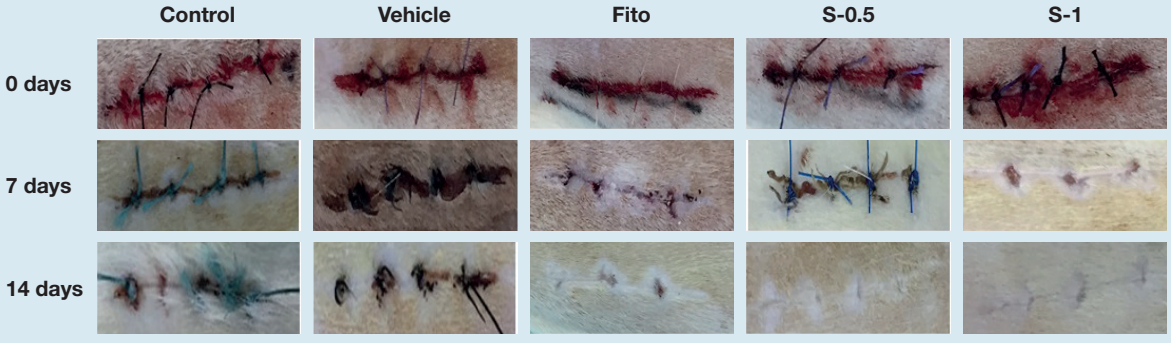
Anaesthesia

Xylazine hydrochloride (10mg/kg) (Rompun; Bayer, Germany) and ketamine hydrochloride (30mg/kg) (Ketanest; Pfizer Inc., US) were injected intraperitoneally to induce anaesthesia.[19]

Wound creation

Whole surgical applications were performed under anaesthesia. The dorsal aspects of anaesthetised animals were shaved and the areas cleaned and sterilised with ethanol (70% volume/volume (v/v)).

Fig 3. Macroscopic view of incision wounds at 0, 7 and 14 days. Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight (w/w)) *Salvia huberi* ointment group; S-1—1% (w/w) *Salvia huberi* ointment group



Excision wound model: to evaluate wound contraction and wound closure time, an open excision-type wound model with 1.5cm diameter was performed in a circular manner using a biopsy punch on the dorsal interscapular region of the rats with removal of the skin. After the operation the excisional wounds were left open.[1] Ointments were topically applied once a day throughout the seven or 14 days. In each group, six randomly selected animals were euthanised on day 8. In each group, the remaining six animals continued to be treated topically with ointments for a total of 14 days. The remaining animals were euthanised on day 15.

Incision wound model: to evaluate biomechanical parameters, an incision-type wound was created (4cm in length) for which three surgical sutures were placed 1cm apart (2cm posterior to the excision-type wound).[26] Test ointments were topically applied once a day throughout the seven or 14 days. In each group, the sutures of the six randomly selected animals were removed on day 7 post-wounding. Animals were euthanised on day 8. The remaining six animals in each group were treated topically with test ointments for a total of 14 days. The sutures were removed on day 14 day post-wounding and the remaining animals were euthanised on day 15.[19,20]

Assessment of wound closure

After wounds had been created under anaesthesia, wounds areas were photographed using a camera (SPOT Insight QE; Diagnostic Instruments, US). On day 0, operated rats were placed individually in the cages. After 7- and 14-day treatments, wounds were rephotographed. Wound areas were evaluated on days 0, 7 and 14 with the SPOT Advanced program (Diagnostic Instruments, US) for determining wound contraction. The length and surface area of both excision and incision wound areas were measured with graph paper seven and 14 days after wound creation (day 0). For each animal, calculation of wound healing ratio was performed using the formula:

$$\text{wound healing ratio}\,(\%) = 100 \times (1 - WS_t/WS_0)^{1,18,19,27}$$

where WS_t = wound size on day t
and WS_0 = wound size on day 0.

Table 2. Gene sequences for *KGF*, *CTGF*, and *GPX1*

KGF	Forward Reverse	GTG GCA GTT GGA ATT GTG GC GAA CAT TTC CCC TCC GCT GT
CTGF	Forward Reverse	ACC TGT GCC TGC CAT TAC AA CTC ACT TCG GTG GGG TGT TT
Beta actin	Forward Reverse	CCC GCG AGT ACA ACC TTC T CGT CAT CCA TGG CGA ACT
GPX1	Forward Reverse	GCT CAC CCG CTC TTT ACC TT GAT GTC GAT GGT GCG AAA GC

Biomechanical study

Tensile Testing System (Ilfa Electronics, Turkey) was used for the biomechanical analyses of skin samples from incisional wounds. An automatic action clamp was used to attach the skin samples to the cross heads of a computer-controlled Tensile Testing System. Using a 1000N load cell, crosshead speed was adjusted from 0–250mm/min. Each skin sample was placed between two clamps, as described in our previous studies.[19,20] Clamped skin was pulled to rupture at a crosshead speed of 25mm/min.[28,29] The data were processed and saved in a Tensile Test System computer. The Tensile Test System software (Ilfa Electronics, Turkey) was used for recording a load–deformation curve. Then, the saved data were evaluated with a Logger Pro Software Programme (V 3.8.3; Vernier Software and Technology, US). The biomechanical parameters (the maximum deformation (du: mm), the maximum load (FU: N), stiffness (S: N/mm), and the energy stored until yield point (U: mJ)) were acquired according to the load–deformation curve. The load–deformation curve was converted to a stress–strain curve and the various parameters (maximum stress (σu: MPa), maximum strain (εU: mm/mm), toughness (u: MPa), and Young's (elasticity) modulus (E: MPa)) were calculated.

Histopathological study

Tissues were fixed in 10% neutral formaldehyde and after the routine tissue follow-up, 4–5µm-thick sections were cut from paraffin blocks and stained with haematoxylin and eosin (H&E)[30] and a Verhoeff-Van Gieson (VVG) stain kit (Atom Scientific, UK)[31] for elastic fibres. Slides were screened under a light microscope

Table 3. Wound healing ratios (% contraction) of excisional and incisional wounds based on treatment. Analysis of variance (ANOVA) performed

Groups		Control	Vehicle	Fito	S-0.5	S-1
Excision wound model						
Application periods, mean±SD (range)	7-Day	40.3±18.8* (9.2–64.2)	42.8±16.8† (24.4–60.6)	69.8±8.6‡ (54.6–80.6)	68.1±16.5‡ (40.4–84.5)	76.6±1.8‡ (74.9–79.6)
	14-Day	75.4±18.6* (37.4–93.0)	84.4±12.6* (72.1–94.2)	99.1±1.1† (97.2–100)	99.3±1.1† (97.1–100)	99.4±0.6† (98.2–100)
Incision wound model						
Application periods, mean±SD (range)	7-Day	31.7±16.4* (16.6–56.8)	46.1±17.1* (29.1–54.2)	84.2±20.2‡ (58.1–92.2)	67.9±23.2‡ (40–90.2)	91.4±3.2‡ (87.2–96.1)
	14-Day	74.1±10.1* (54.1–87.2)	73.8±12.4‡ (61.8–94.6)	99.0±1.1‡ (97.3–100)	99.9±0.1‡ (99.7–100)	97.0±2.5‡ (94–100)

*p>0.05; †p<0.05; ‡p<0.01. Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight) *Salvia huberi* ointment group; S-1—1% (weight/weight) *Salvia huberi* ointment group; SD—standard deviation

Table 4. Skin biomechanical parameters of incision wound models

Groups	Skin biomechanical parameters: Energy absorption capacity (U: mj)	Stiffness (S: N/mm)	Maximum load (Fu: N)	Maximum deformation (du: mm)	Young's (elasticity) modulus (E: MPa)	Toughness (u: MPa)	Maximum stress (σu: MPa)	Maximum strain (εu: mm/mm)
7-day applications, mean±standard deviation								
Control	88.54±24.75	4.23±1.8	19.08±4.89	11.38±2.32	0.05±0.02	0.26±0.04	0.10±0.02	4.05±1.02
Vehicle	86.36±32.12	3.08±1.3	19.29±4.01	11.42±2.21	0.04±0.02	0.20±0.02	0.10±0.04	4.25±1.21
Fito	120.90±15.50*‡	3.32±1.21	21.18±2.92	14.75±2.12*‡	0.03±0.01	0.29±0.03*‡	0.11±0.03	5.82±1.48*‡
S-0.5	41.56±21.10†	5.32±1.09	18.96±5.98	9.62±2.37†	0.41±0.29†	0.06±0.07*†‡	0.09±0.03	3.89±1.16†
S-1	79.03±21.34†	3.48±1.00	21.55±4.36	12.08±1.76†	0.04±0.01	0.20±0.05†	0.11±0.02	4.95±0.98†
14-day applications, mean±standard deviation								
Control	175.42±41.17	3.53±2.12	26.92±6.91	15.85±4.15	0.05±0.03	0.40±0.16	0.12±0.04	6.89±2.63
Vehicle	99.23±32.43	4.08±2.28	25.42±5.84	12.53±3.52	0.04±0.01	0.29±0.22	0.14±0.05	4.98±1.49
Fito	169.32±66.43	4.21±2.17	29.04±5.96	13.28±4.01	0.04±0.03	0.38±0.28	0.14±0.02	6.24±1.65
S-0.5	65.12±42.64*†	7.14±4.61	24.83±13.83	10.13±3.14	0.16±0.11*†‡	0.08±0.06†	0.12±0.07	4.05±1.57
S-1	134.44±35.53	4.43±1.45	28.45±4.75	14.08±2.98	0.05±0.02	0.34±0.09	0.14±0.02	6.07±1.49

*significantly different from Control group (p<0.05); †significantly different from Fito group (p<0.05); ‡significantly different from Vehicle group (p<0.05). Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight) *Salvia huberi* ointment group; S-1—1% (weight/weight) *Salvia huberi* ointment group

(Zeiss, Germany). Wound healing was examined in terms of re-epithelialisation, angiogenesis, thickness of granulation tissue, extent of dermal inflammation and amount of early collagen present. Histological scoring was performed by two blinded observers with a semi-quantitative system as follows: re-epithelialisation 0=absence; 1=mild (<50%); 2=moderate (>50%); and 3=complete re-epithelialisation.[32] Angiogenesis was scored as: 0=absence; 1=mild (<5 vessels per one field); 2=moderate (6–10 vessels per one field); and 3=marked (>10 vessels per one field). Inflammatory cell infiltration and granulation tissue were scored as: 0=absence; 1=mild; 2=moderate; and 3=marked.[33]

Biochemical analysis

Hydroxyproline measurement: tissues were dissolved in 1ml of 50% (v/v) isopropyl alcohol and hydrolysed. Then, 25µl of each sample was mixed with chloramine-T. Ehrlich's reagent (1ml) was added and the sample was incubated for 90 minutes at 50°C. After the reaction, colour changes were measured at 560nm using a spectrophotometer (UV 1601; Shimadzu, Japan). Under the same conditions, a diverse concentration series of hydroxyproline standards (0.2–1.6µg) were also tested. Sample concentrations were calculated according to the standard curve and results are represented as µg/mg dry weight of tissue.[34]

Malondialdehyde (MDA) measurement: thiobarbituric acid-TBARS assay kit (Cayman Chemical, US) was used to measure tissue MDA levels (530nm). Results are represented as nmoles/g tissue.[35]

Nitric oxide (NOx) measurement: nitrate–nitrite assay

Table 5. Histological scores of groups in excisional wound model. Analysis of variance (ANOVA) performed

Application period	Group	Parameter (mean±standard deviation): Re-epithelialisation	Granulation tissue	Angiogenesis	Inflammation
7-Day	Control	0.16±0.16	0.83±0.30	0.33±0.21	2.17±0.16
	Vehicle	0.16±0.12	–	0.33±0.21	2.00±0.25
	Fito	1.50±0.22*	1.00±0.36	1.66±0.21*	0.33±0.21†
	S-0.5	1.83±0.31*	–	2.33±0.21*	0.83±0.31†
	S-1	2.33±0.21*	0.66±0.21*	2.33±0.33*	0.50±0.22†
14-Day	Control	0.50±0.22	0.53±0.21	0.50±0.22	1.83±0.40
	Vehicle	0.50±0.22	0.33±0.21*	0.50±0.22	2.00±0.25
	Fito	2.16±0.16†	–	1.50±0.22*	0.16±0.16†
	S-0.5	2.50±0.22*	0.33±0.21*	2.50±0.34*	1.00±0.36†
	S-1	2.66±0.21†	–	2.40±0.24†	0.33±0.21†

*p<0.05; †p<0.001. Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight) *Salvia huberi* ointment group; S-1—1% (weight/weight) *Salvia huberi* ointment group. All results belong to control compared to the other groups

Table 6. Comet and micronucleus assay results per treatment and length of application. Analysis of variance (ANOVA) performed

	Groups					
	Control	Vehicle	Fito	S-0.5	S-1	Positive control (MMC, 2mg/kg)
7-day application, mean±standard deviation						
GDI	368.1±7.1	178.8±4.0†	237±19.1*	27.33±7.24‡	31.6±8.25‡	237.0 ± 15.6*
DCP	97.0±1.52	44.9±1.72*	18.50±1.95‡	6.00±2.06‡	4.66±0.84‡	84.8 ± 3.2
MN/2000	3.66±0.33*	3.06±0.16†	1.83±0.16‡	2.16±0.30‡	1.50±0.22‡	5.3±0.3
14-day application, mean±standard deviation						
GDI	346±12.7	203.2±5.1*	83.35±2.6†	27.83±5.51‡	20.6±3.04‡	237.0 ± 15.6*
DCP	95.8±4.16	56.3±2.44*	8.00±1.26‡	4.16±0.54‡	4.00±0.89‡	84.8 ± 3.2
MN/2000	4.33±0.42*	3.42±0.16†	1.66±0.21‡	2.00±0.25‡	1.50±0.22‡	5.3±0.3

*p<0.05; †p<0.01; ‡p<0.001; GDI—genetic damage index; DCP—damaged cell percent; MN—micronucleus; MMC—mitomycin-C; Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight) *Salvia huberi* ointment group; S-1—1% (weight/weight) *Salvia huberi* ointment group

kit (Cayman Chemical, US) was used to measure tissue NOx level (540nm). Results are represented as µM/g tisssue.[36]

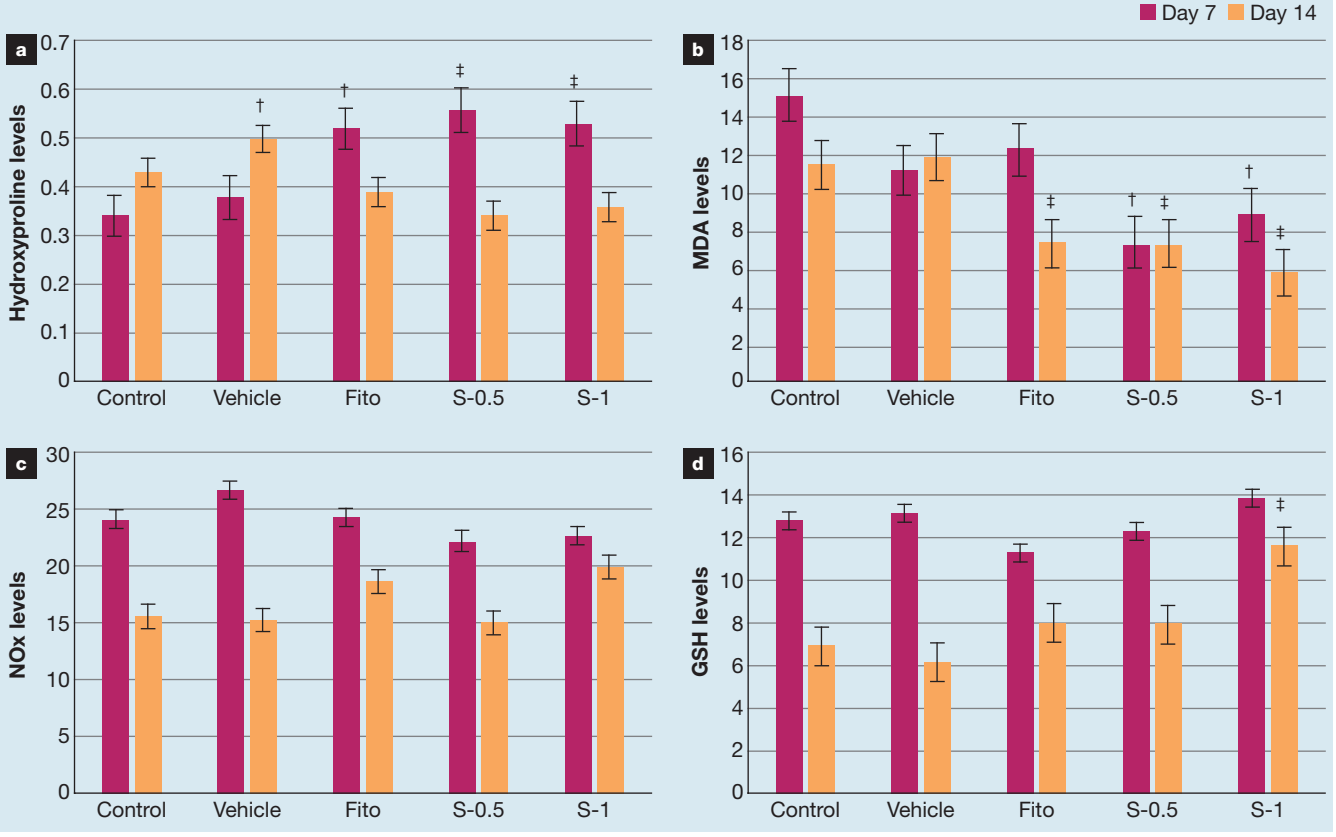
Glutathione (GSH) measurement: GSH assay kit (Cayman Chemical, US) was used to measure tissue GSH level (410nm). Results are represented as nmoles/mg protein.[37]

Measurement of protein levels: the tissue protein level was measured based on the Bradford technique.[38] Results are represented as µg/g total protein.

Genotoxicologic analysis

Comet and micronucleus (MN) assays were performed in rat peripheral blood samples after seven and 14 days'

Fig 4. Biochemical analysis results of all tested groups. Analysis of variance (ANOVA) was performed, measuring the mean±standard deviation. Hydroxyproline levels **(a)**; malondialdehyde (MDA) levels **(b)**; nitric oxide (NOx) levels **(c)**; glutathione (GSH) levels **(d)**. Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight (w/w)) *Salvia huberi* ointment group; S-1—1% (w/w) *Salvia huberi* ointment group. †p<0.05; ‡p<0.01



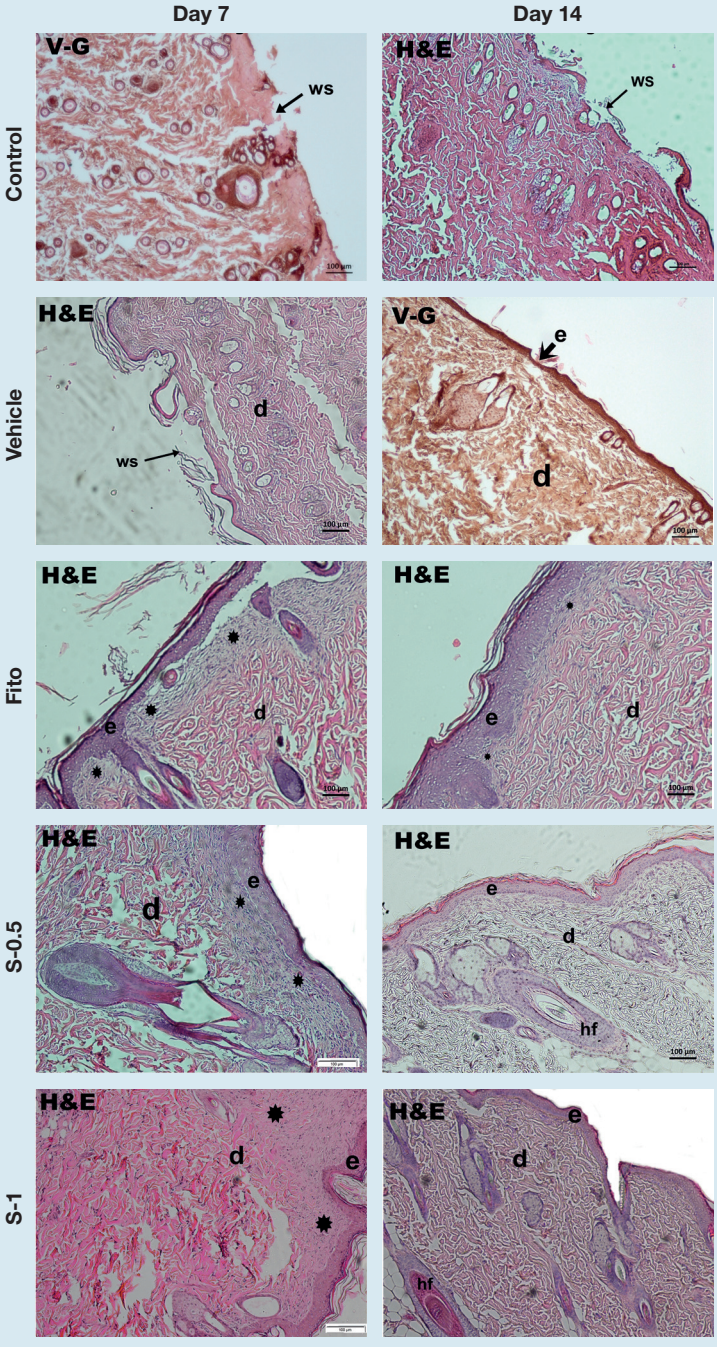


Fig 5. Histological view of excisional wound healing in all groups at day 7 and day 14. Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight (w/w)) *Salvia huberi* ointment group; S-1—1% (w/w) *Salvia huberi* ointment group; *granulation tisssue with inflammatory cells; d—dermis; e—epidermis; H&E—haematoxylin and eosin; hf—hair follicle; V-G—Verhoeff-Van Gieson stain; ws—wound surface

treatment, according to the method extensively described in our previous studies.[19,20]

The comet assay: genetic damage was determined by evaluating the genetic damage index (GDI) and damaged cell percent (DCP) in the comet test. Mitomycin-C (MMC) was used as a positive control. Each test group was studied with six parallel samples.

- GDI: The results represented as arbitrary units (AU) expressed the extent of DNA damage. Results were calculated according to the formula given below:[19,20]

AU values indicating the comet assay scores were: UD (undamaged, 0); type 1 (low level of damage, 1); type 2 (moderately damaged, 2); type 3 (highly damaged, 3); type 4 (very highly damaged, 4).

GDI = ((0×type 0) + (1×type 1) + (2×type 2) + (3×type 3) + (4×type 4)]

DCP = type 2 + type 3 + type 4

The MN assay: 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 MN assay results were determined based on the colour changes (immature erythrocytes, i.e., PCEs were determined according to their orange–red colour, micronuclei by their yellowish colour, and mature erythrocytes by their green colour).[19,20]

Determination of growth factors' expression levels

The expression levels of *GPX1*, *CTGF* and *KGF* genes were analysed by a quantitative polymerase chain reaction (qPCR) approach. Sequences of gene-specific primers are given in Table 2. Total RNA isolations were performed from the tissues with GeneJET RNA Purification Kit (ThermoFisher Scientific Inc., US). RNA samples were stored at –80°C until use. RNA samples were converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, US). Gene expression analysis was performed with Real Q Plus 2x Master Mix Green (Ampliqon, Denmark) using the Rotor-Gene Q real-time PCR instrument (Qiagen, Germany).

Statistical analysis

Statistical analysis of data was performed with SPSS 20.0 (IBM Corp., US), represented as the mean±standard deviation (SD), and evaluated with analysis of variance (ANOVA) Tukey post hoc test. The plots were designed by GraphPad Prism version 6.0 (GraphPad Software, US). In histopathological analyses, normal distribution featuring data (Shapiro–Wilk test, $p \geq 0.05$) was performed. Normality of the data was confirmed with the Kolmogorov–Smirnov D test ($p \geq 0.05$) for genotoxicity studies. Data having a normal distribution were analysed by Student t-test. A p-value of <0.05 was considered statistically significant.

Results

Wound closure results

Wound healing ratios for excision and incision wound models are presented in Table 3. Macroscopic views of

excision and incision wounds at 0, 7 and 14 days are shown in Figs 2 and 3, respectively. For excisional wounds, on day 7 and day 14, wound healing ratios of the S-1, S-0.5 and Fito groups were statistically significantly higher than for the Control and Vehicle groups ($p<0.01$ and $p< 0.05$, respectively). According to the results of both application periods, the highest wound healing ratios were observed in the S-1 group (76.6±1.8% and 99.4±0.6%, for samples on the seventh and 14th days, respectively).

For incisional wounds, on day 7 and day 14, wound healing ratios of S-1, S-0.5, and Fito groups were statistically significantly higher than Control and Vehicle groups ($p<0.01$). On day 7, S-1 was found to be the most effective, with a wound healing ratio of 91.4±3.2%. On day 14, wound healing ratios of the Fito, S-0.5 and S-1 groups were 99.0±1.1%, 99.9±0.1%, and 97.0±.0%, respectively.

Biomechanical results

Biomechanical parameters were studied on incision wound samples and results are given in Table 4. On day 7, the Fito group showed a statistically significantly greater toughness, energy absorption capacity and maximum strain values than the Control and Vehicle groups ($p<0.05$; all comparisons). The S-0.5 group showed a statistically significantly higher elasticity value and the S-0.5 and S-1 groups showed lower maximum deformation values than the Fito group ($p<0.05$). On day 14, the S-0.5 group showed a statistically significantly higher elasticity value (0.16±0.11) than the Control (0.05±0.03), Vehicle (0.04±0.01) and Fito (0.04±0.03) groups ($p<0.05$; all comparisons). Also, the S-0.5 group showed a statistically significantly lower energy absorption capacity value (65.12±42.64) than the Control and Fito groups ($p<0.05$; both comparisons) and toughness value than the Fito group ($p<0.05$). No statistically significant difference was observed between the other groups.

According to the results of both application periods, 1% (w/w) *Salvia huberi* ointment administration resulted in better skin quality with higher energy absorption capacity, elasticity, maximum load capacity, toughness, maximum stress and maximum strain capacities, and lower stiffness and maximum deformation than 0.5% (w/w) *Salvia huberi* ointment administration. Moreover, on both days 7 and 14, skin quality of the Fito group was better than the 1% (w/w) *Salvia huberi* ointment group, with higher energy absorption capacity, toughness and maximum strain capacity, and lower stiffness.

Biochemical results

On day 7, hydroxyproline content was statistically significantly higher in the S-0.5, S-1 ($p<0.01$; both comparisons) and Fito ($p<0.05$) groups than in the Control and Vehicle groups. On day 14, hydroxyproline content of the Vehicle group was statistically significantly higher than in the S-0.5 and S-1 groups ($p<0.05$; both comparisons). There were no significant differences between the other groups ($p>0.05$) (Fig 4a). On day 7, MDA levels were statistically significantly lower in the S-0.5 and S-1 groups than in the Control ($p<0.01$; both comparisons), Vehicle and Fito groups ($p<0.05$; all comparisons). The MDA levels of the Fito and Vehicle groups were also statistically significantly lower than those of the Control group ($p<0.05$; both comparisons). On day 14, MDA levels of the Fito, S-0.5, and S-1 groups were statistically significantly lower than those of the Control and Vehicle groups ($p<0.01$; all comparisons) (Fig 4b). On both day 7 and day 14, no statistically significantly differences were found between NOx levels of the studied groups ($p>0.05$) (Fig 4c). On day 7, no statistically significant differences were found between GSH levels of the studied groups ($p>0.05$) while on day 14, the GSH level of the S-1 group was statistically significantly higher than the other groups ($p<0.01$; all comparisons) (Fig 4d).

Fig 6. Comet results of groups. Type I—low level of damage; Type III—highly damaged; Type IV—very highly damaged

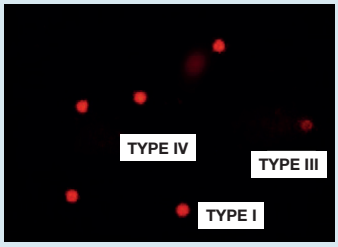
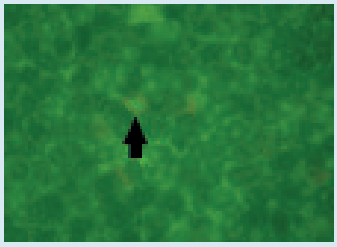


Fig 7. Control group micronucleus. Micronucleated cell (arrow) with orange coloured cell membrane



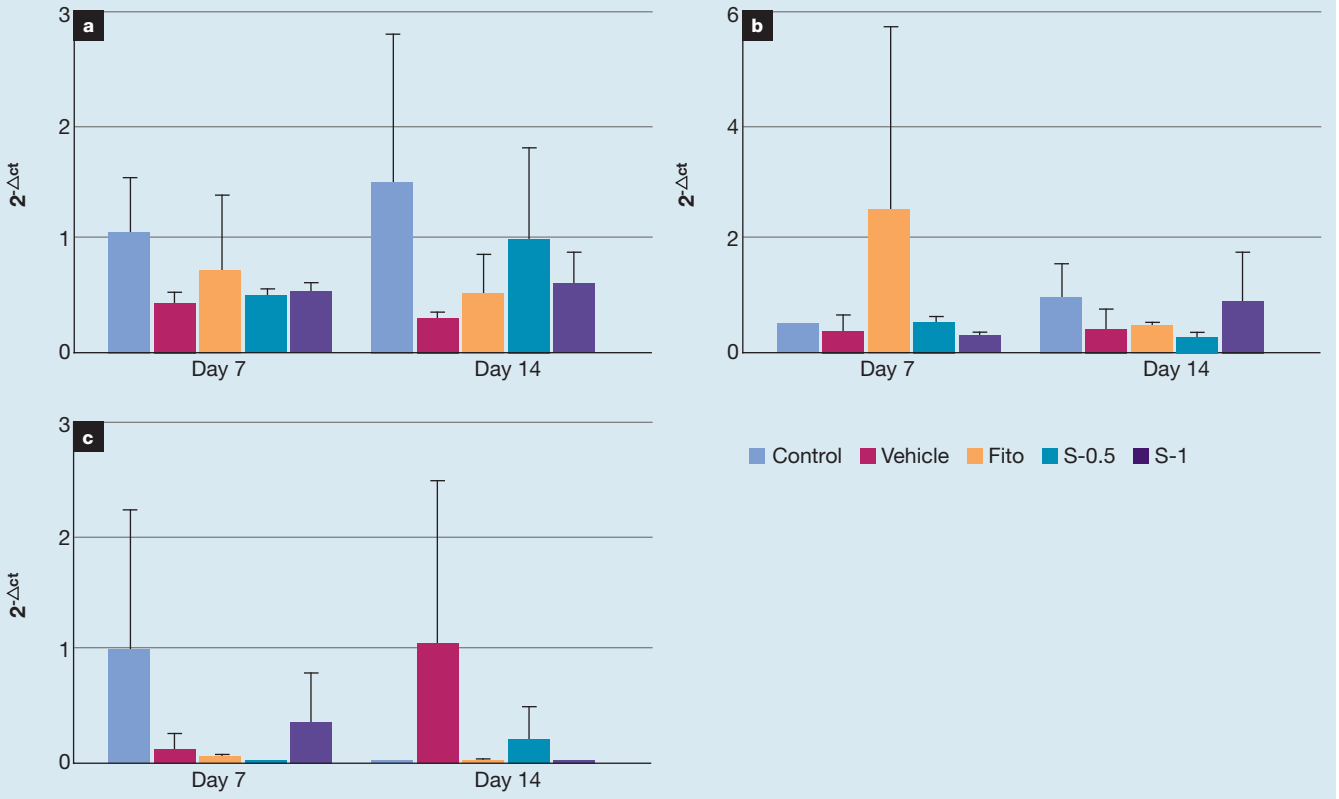
Histopathological evaluation

Histological results in the excisional wound model are presented in Table 5 and Fig 5. Application of *Salvia huberi* ointment (0.5% and 1% (w/w)) markedly induced re-epithelialisation and angiogenesis, deposition of collagen fibres, and also significantly reduced granulation tissue thickness and inflammation when compared with the Control and Vehicle groups.

On day 7, the wound surface was still unhealed, re-epithelialisation had not yet started and loosely distributed collagen fibres were observed in both the Control and Vehicle groups. On day 14, re-epithelialisation had started, but the wound surface had not completely closed and the dermis was observed as immature in the Control group. Deep papillae were not observed. Similar results were observed in the Vehicle group—collagen fibres in the dermis were very loose and sparsely arranged. Epidermis formed again, but keratinisation was weak and quite thin. Neither papillae nor hair follicles were well developed in both the Control and Vehicle groups.

In *Salvia huberi* (1% and 0.5% (w/w)) and Fito cream groups, re-epithelialisation had markedly increased by both day 7 and day 14 compared with the Control and Vehicle groups ($p<0.05$; all comparisons). Thick collagen

Fig 8. Bar graph of *GPX*, *CTGF* and *KGF* gene expression results in all studied groups. *GPX* gene expression result **(a)**; *CTGF* gene expression result **(b)**; *KGF* gene expression result **(c).** Control—Control group; Vehicle—Vehicle group; Fito—Fito cream group; S-0.5—0.5% (weight/weight (w/w)) *Salvia huberi* ointment group; S-1—1% (w/w) *Salvia huberi* ointment group



fibres were observed in the dermis, and healing of the dermis had almost completed in the *Salvia huberi* ointment groups (1% and 0.5% (w/w)) when compared with the Control and Vehicle groups on both day 7 and day 14 ($p<0.001$; all comparisons).

By day 7, both the *Salvia huberi* (1% (w/w)) and Fito cream groups showed abundant accumulation of fibroblasts, inflammatory cells, capillary vessels and collagen fibrils in the granulation tissue. By day 14, granulation tissue seemed to have disappeared in the *Salvia huberi* (1% and 0.5% (w/w)) groups, and been replaced by mature dermis. Re-epithelialisation had completed, and the dermis had almost completely healed. Hair follicles were also well developed in these groups.

Genotoxicology results

Genotoxic study results are presented in Table 6 and Figs 6 and 7. The results showed that after seven days of application, administration of *Salvia huberi* ointments (1% and 0.5% (w/w)) led to a reduction in GDI and DCP values, and MN frequency, that were statistically significant when compared with the Control group ($p<0.001$; all comparisons). In the S-0.5 and S-1 groups, GDI and DCP values and MN frequency were 27.33±7.24, 6.00±2.06 and 2.16±0.30, and 31.6±8.25, 4.66±0.84 and 1.50±0.22, respectively. In the Fito group, GDI ($p<0.05$), and DCP values and MN frequency ($p<0.001$; both comparisons), were statistically different than in the Control group.

After 14 days of application, similar results were observed for GDI (27.83±5.51 and 20.6±3.04) and DCP (4.16±0.54 and 4.00±0.89) values, and MN frequency (2.00±0.25 and 1.50±0.22) in the *Salvia huberi* ointment (0.5% and 1% (w/w)) treatment groups ($p<0.001$; all comparisons). The highest GDI and DCP values and MN frequency were observed in the Control (346±12.7, 95.8±4.16 and 4.33±0.42, respectively) and Vehicle (203.2±5.1, 56.2±2.44 and 3.42±0.16, respectively) groups. In the Fito group, GDI ($p<0.01$), and DCP values and MN frequency ($p<0.001$; both comparisons) were statistically different than in the Control group.

Results of growth factors' gene expressions

The results of gene expressions (*GPX1*, *CTGF*, and *KGF*) taken from skin tissues are presented in Fig 8a–c. *GPX1* gene expression levels on day 14 were highest in the Control group and lowest in the Vehicle group. *CTGF* gene expression levels were highest in the Control group on day 14 and the Fito group on day 7. *KGF* gene expression levels were highest on day 14 in the Vehicle group and lowest in the S-0.5 group on day 7.

Discussion

In the present study, we evaluated the wound healing efficacy of ointments prepared from different concentrations (1% and 0.5% (w/w)) of ethanol extract obtained from aerial parts of *Salvia huberi* on excisional and incisional wounded diabetic rats using macroscopic, biomechanical, biochemical, histopathological, genotoxic and gene expression methods for both seven and 14 days.

In the literature, diabetic skin wounds untreated for two weeks exhibited ulceration, inflammation, defective granulation tissue formation and significant decrease in collagen deposition.[39] It was also reported that in diabetes impairment of new blood vessel formation slows down the healing process. Diabetes is well known to cause a delay in wound healing phases, especially in the inflammation phase, by waiving the immunity,[40] and the proliferative phase with dysfunction in fibroblasts, collagen deposition and defective neovascularisation, and also the remodelling phase with deficient tissue structure establishment.[41] Diabetic wounds are associated with deficiency in extracellular matrix production, dermal protein structure and proteolysis. Decreased collagen content and a deficient healing process are also seen in diabetic wounds.[5] In this present study, compared with previous studies, we observed similar results, with an incomplete macroscopic wound healing process and serious re-epithelialisation and collagen fibre deposition delays on the seventh and 14th days.

Beside important parameters such as wound closure, wound contraction and functional barrier re-organisation,[1] type of damage (excision, burn, incision etc.)[42] plays a significant role in the wound healing process. This explains different wound healing ratios of excision and incision wounds in the present study. Comparison of *Salvia huberi* ointment (1% and 0.5% (w/w)) results indicated that 1% (w/w) *Salvia huberi* ointment appeared to be more effective than 0.5% (w/w) *Salvia huberi* ointment on day 7. On day 7, wound healing ratios in 1% (w/w) *Salvia huberi* ointment treatment groups were 91.4% for incision wounds and 76.6% for excision wounds. According to the macroscopic study, results of both days 7 and 14, for both concentrations (1% and 0.5% (w/w)) of *Salvia huberi* ointments, were found to be as effective as the reference drug, Fito cream.

In the wounded area, skin quality is determined by measuring skin biomechanical parameters. For this reason, skin biomechanical parameters are a significant reflection of the subdermal organisation of newly synthesised collagen fibrils in deposited collagen.[43] According to the biomechanical study results of both day 7 and day 14, administration of 1% (w/w) *Salvia huberi* ointment and Fito cream resulted in better skin quality with higher values for energy absorption capacity, toughness, maximum load, maximum stress and maximum strain capacity and lower values for stiffness than those for 0.5% (w/w) ointment administration. Comparison of biomechanical parameters of both application periods indicated that long-term 1% (w/w) *Salvia huberi* ointment application to diabetic skin wound contributed to healing because parameters obtained here were similar to the reference drug, Fito cream.

An increase in hydroxyproline levels of healing tissues is a demonstration of rapid wound healing. Hydroxyproline measurement is an acceptable procedure to predict the amount of collagen synthesis.[44]

In the present study, *Salvia huberi* ointments (1% and 0.5% (w/w)) and Fito cream administration on day 7 contributed to regulation of collagen fibrils and collagen formation by increasing the hydroxyproline levels; but on day 14, decreasing hydroxyproline levels were seen in the same groups. Because of increasing reactive oxygen species (ROS) production or decreasing antioxidant scavenging efficiency, increased MDA levels were found in wound tissues of diabetic rats.[45] When compared with control groups, decreased MDA levels were observed in *Salvia huberi* (1% and 0.5% (w/w)) ointment and Fito cream-treated groups on both day 7 and day 14.

NOx has beneficial effects on angiogenesis, inflammation, cell proliferation, matrix deposition and remodelling; therefore, it plays crucial roles in the repair process of wounds. Studies on excisional wounds showed that urinary nitrate excretion was increased in the early stages (1–5 days) of healing, and then NOx levels decreased. In incisional wounds, lack of NOx is related to impaired wound healing, decreasing collagen deposition and breaking mechanical strength. Comparison of impaired wound healing and decreased cutaneous NOx levels in diabetic wounds indicated that there was a strong correlation between them. NOx levels of STZ-induced diabetic animals were found to be significantly decreased.[46] When the day 7 and day 14 results of the present study were compared, NOx levels of all studied groups were higher on day 7 than on day 14.

The wound healing process is associated with antioxidant activity, on account of increasing colloid synthesis and/or removing free oxygen radicals.[47] Also, antioxidants are known to be important in the healing of hard-to-heal wounds with the suppression of the ROS pathway.[48] Antioxidant enzyme levels decreased in serum with oxidative stress in diabetic animals. A non-enzymatic antioxidant, GSH regulates the early phases of healing in wound tissues; for this reason, lack of GSH may cause delay in the healing process in diabetic wounded rats.[49] Increasing GSH levels were observed in the 1% (w/w) *Salvia huberi* ointment-treated group on day 7, and in both the *Salvia huberi* ointments (1% and 0.5% (w/w)) and Fito cream-treated groups on day 14.

It has been reported that the levels of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were increased for the suppression of radicals after plant-based extract

application, and also the wound healing rate was increased accordingly.[50] Antioxidant GPX enzyme activities increase in the presence of oxidative stress.[51] GPX levels also increased with oxidative stress in wound tissue.[52] In our study, we observed in the Control group that *GPX1* expression levels continued to be higher when the wound healing process was delayed and prolonged compared with the other groups. In addition, it was determined that *GPX1* mRNA levels were decreased after *Salvia huberi* ointment (1% and 0.5% (w/w)) administration when compared with the control. This reduction may be caused by the decrease in the load on the enzymatic antioxidant system, as the phytochemicals in the extract help to maintain antioxidant balance in the cells.[53] *Salvia huberi* (1% and 0.5% (w/w)) and Fito cream applications positively affected wound healing at the tissue level. Gradual improvement was observed in the scar tissue on both days 7 and 14 in both the *Salvia huberi* and Fito cream groups. *Salvia huberi* was able to markedly accelerate the delayed wound healing caused by diabetes.

Growth factors, with their ability to communicate between cells, are involved in inflammation, tissue repair and regeneration following tissue damage.[54,55] All phases of wound healing are under the control of growth factors, represented as described previously.[56–58] Fibroblast growth factor/keratinocyte growth factor-1 (KGF1 or FGF7) is one such growth factor. It is required for re-epithelialisation and is involved in the migration and proliferation of keratinocytes.[59] It has been reported that KGF is induced after injury and its expression in skin tissue is increased.[60] KGF is involved in wound repair by binding to epithelial cell receptor (FGFR2). Although many studies have indicated the levels of KGF in wound tissue, studies indicating the alterations in KGF expression after the application of plant-derived extracts are limited. This constitutes another novel point of our study. In our study, KGF expression was significantly decreased in the 0.5% (w/w) *Salvia huberi* ointment group compared with the Control group on day 7. However, on day 14, KGF expression levels were decreased in the Control group but increased in the 0.5% (w/w) *Salvia huberi* ointment group. In previous studies, it has been reported that the healing process has reached the highest level at the end of the first 24 hours, after which it started to decrease, and then began to increase again from day 15.[54,60] In the study by Werner et al.,[60] the KGF level increased in the injured tissue in the first 24 hours compared with normal tissue. Although there was a decrease in KGF level on day 7, it was still highly expressed (100-fold) compared with the Control. In this context, we suggest that the decrease in KGF levels in both the 1% and 0.5% (w/w) *Salvia huberi* ointment groups on days 7 and 14 compared with the Control group may be an indicator of the completed healing process at the wound site. We examined connective tissue growth factor (CTGF), which is a member of the CCN family (connective tissue growth factor, cysteine-rich protein and nephroblastoma) and consists of 349 amino acids.[61] CTGF is known to be involved in cell migration and differentiation, and in proteoglycan synthesis.[62] In the present study, CTGF levels were not different between the Control and Vehicle groups in the first 7 days but, on day 14, the CTGF level had decreased significantly in the 0.5% (w/w) *Salvia huberi* ointment group compared with the Control group. We propose that the reason for the decreases in CTGF and KGF levels seen by days 7 and 14 were due to the rapid completion of the healing process.

The genotoxic effects of STZ in rats of different ages have been studied previously and results have indicated that STZ causes MN induction in the bone marrow of adult rats, and in the peripheral blood and bone marrow of neonatal and young rats. Moreover, STZ (30mg/kg) application for three days induced significant DNA damage in the bone marrow and peripheral blood of young rats.[63] In this present study, after both 7 and 14 days of application, the DCP and GDI values of *Salvia huberi* ointment (1% and 0.5% (w/w)) treatment groups and MN frequency of *Salvia huberi* ointment (1% and 0.5% (w/w)) and Fito cream treatment groups were lower than in the Control and Vehicle groups.

Some *Salvia* species have been investigated for their wound healing potential: *Salvia virgata* Jacq. has been used to expedite wound healing, against skin diseases and blood cancer.[64] Angiogenic activity of *Salvia miltiorrhiza* Bunge has been reported in endothelial cells of human umbilical cord.[65] Wound healing effects of hydroethanolic extract of *Salvia officinalis* L. leaves on excision and incision wounds,[66] methanolic extract of *Salvia splendens* Sellow ex Roemer & J. A. Schultes leaves on excision and incision wounds,[18] and methanolic extract of aerial parts of *Salvia multicaulis* Vahl. on excision wounds have been indicated.[67] Our previous studies on ethanol extracts of *Salvia kronenburgii* Rech. f., S. euphratica Montbret, Aucher & Rech. f. var. euphratica,[19] and *Salvia hypargeia* Fisch. & Mey.[20] revealed strong wound healing effects on incision and excision wound models of STZ-induced diabetic rats. Also, our group showed that ethanolic extract of *Salvia huberi* had antimicrobial activity against several microorganisms, including *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Aeromonas hydrophila*, *Acinetobacter baumannii*, *Mycobacterium tuberculosis* H37Rv, *Candida parapsilosis*, *Candida glabrata* and *Candida tropicalis*.[18] *Staphylococcus* and *Pseudomonas* are responsible for microbial infections in hard-to-heal wounds,[68] and *Acinetobacter baumannii* is responsible for infections in surgical wounds.[69] We propose, therefore, that in this study the ethanol extract of *Salvia huberi* has contributed to wound healing due to its antimicrobial properties, as demonstrated in our previous study.[13]

Limitations

The small sample size, and the physiological and anatomical differences between rats and humans should be considered as limiting factors in this study. Therefore, the effect in humans requires further investigation.

Conclusion

Perfect wound healing is described as complete closure in a short time with no side-effects. According to the macroscopic, biomechanical, biochemical, histopathological, genotoxic and gene expression results, application of *Salvia huberi* ointments promoted visible improvement in the healing process, with complete re-epithelialisation and tissue integrity. In spite of the negative effects of diabetes, the wound healing phenomenon was well organised and application of *Salvia huberi* ointment showed strong healing in diabetic rats. To the best of our knowledge, this is the first report describing wound healing activity of *Salvia huberi*. Due to their minimal side-effects and dominant positive effects on human health, plant-derived medicinal products are an alternative to synthetic drugs. Our results show that endemic *Salvia huberi*, which is abundant in nature, seems to be a promising wound healing agent for the treatment of impaired and persistent diabetic wounds. **JWC**

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Reflective questions

- What is the role of plant extracts in the healing of diabetic wounds?
- How do the higher or lower concentrations of the plant extract affect wound healing?
- How is *Salvia huberi* ethanol extract useful in the treatment of excisional and incisional wounds?

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