

Evaluation of Wound Healing Activity of Hydromethanolic Crude Extract and Solvent Fractions of the Root of *Verbascum Sinaiticum* Benth. (*Scrophulariaceae*) in Swiss Albino Mice

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Background: The roots of *Verbascum sinaiticum* have been used traditionally for the management of wound in different regions of Ethiopia. Despite the presence of several claims and in vitro studies regarding its role in wound healing, no scientific studies have been conducted so far. Therefore, this study aims to scientifically evaluate the wound healing activities of the crude extract and solvent fractions of the roots of *Verbascum sinaiticum* in Swiss albino mice.

Methods: The dried root powder of *Verbascum sinaiticum* was extracted using 80% methanol by maceration technique. This was then fractionated with chloroform, ethyl acetate, and water. These extracts were formulated as ointment at 5% and 10% concentration by using simple base. Acute dermal toxicity was performed on mice. The wound healing potential was evaluated using excision, incision, and burn wound models.

Results: In excision wound, 10% and 5% of crude extract ointment provided a significant ($P < 0.001$) percentage of contraction starting from day 4 and day 6 onwards respectively. Moreover, the rate of epithelialization was significantly ($P < 0.001$) improved in 10% crude extract. In burn wound, 10% and 5% crude extract showed significant ($P < 0.001$) wound contraction starting from day 4 and 8 onwards respectively. In both excision and burn wounds, a moderate concentration of fibroblast proliferation and collagen deposition was observed on the 10% crude extract. The 5% and 10% aqueous and ethyl acetate fractions produced a significant ($P < 0.001$) percentage of wound contraction and shortening of epithelialization at different time points compared to simple ointment.

Conclusion: The results of this study demonstrated that the 80% methanolic crude extract, aqueous and ethyl acetate fractions of *Verbascum sinaiticum* root have wound healing potential which assimilates its traditional use.

Keywords: wound healing, crude extract, solvent fraction, *Verbascum sinaiticum*

Background

A wound is defined as a disruption of anatomical, cellular, or functional integrity of tissue in the presence or absence of microbial infection.¹ It can be classified based on the type of injury (burn, incised, and crushed), timing (acute, early, and late), and depth (superficial, dermal, and full-thickness).² Wounds impose a huge clinical and financial burden to healthcare systems around the globe by imposing a significant effect in reducing the quality of life in those who are challenging with the wound.³ Worldwide, the amount of money required for wound care has been increasing, which makes institutions to acquire a more relevant and consistent ways to improve wound assessment.⁴ Approximately 2% of the world population who are admitted to the hospital has chronic wounds.^{5,6} According to the study done in 2017, at Gondar university hospital, 25% emergency visits were due to injury.⁷ Another studies which assessed the epidemiology of burn injuries from 2000–2018 reported that burn injuries in Ethiopia accounts for about 1.5% to 9%.⁸



Figure 1 The photograph of *Verbascum sinaiticum* January 2021, Maraki campus, University of Gondar, Gondar, Amhara regional state, Ethiopia.

Medicinal plants are important in the management of different type of diseases which affect humans. Their parts have been utilized in different techniques to get their active constituent.⁹

Verbascum sinaiticum is one species of *Verbascum* which is popularly known in Ethiopia by the name *kutitina* (Amharic), *ye aheya joro* (amharic), *Gurra Harree* (Afan Oromo), and *Tirnake* (Tigrigna) (Figure 1).¹⁰

For wound healing purposes, in debark district, North Gondar, Ethiopia, the roots of *V. sinaiticum* are crushed, powdered, mixed with butter, and creamed on the affected part¹⁰ and in dera district, South Gondar zone, Amhara region, Ethiopia, the dry root is ground and applied on the wound.¹¹ It is also used to treat the wound in Ensaro District, North Shewa Zone, Amhara Regional State, Ethiopia.¹² The leaves are also used for fire burn and external wounds.¹³

Scientifically investigated in vitro studies have reported that methanol extract of *V. sinaiticum* root shows outstanding activity against *S. aureus* and *S. epidermides*¹¹. Another in vitro study on *V. sinaiticum* reported its antioxidant activity.¹⁴

Rationale of the Study

Wound has been still a challenge for medical care system and it imposes a great problem with in the society by causing morbidity and mortality and its cost of care has been increasing. Current treatment strategies are unable to heal the wound completely and the emergence of drug resistance strains as well as the limited tolerability of the systemic and topical drugs and their cost bends the focus of the treatment of wound in to traditional medicine or medicinal plants. *Verbascum sinaiticum*, one of the known plant in Ethiopia, its root has been claimed by traditional healers for the management of wound. Different in-vitro studies showed that the plant has a good antimicrobial activity, and the presence of different chemical components. Therefore, based on its use in folk medicine for wound healing and its invitro antibacterial activities and secondary metabolites, it is reasonable to conduct an invivo evaluation of its healing potential to provide scientific basis for its use in traditional medicine as a healing agent. There is also a need of scientific validation, standardization and safety evaluation to protect consumers from overdose as well from low therapeutic dose. The results of this study may provide baseline information for future studies intended at identification, isolation, purification and efficacy determination of the active compound for synthesis of lead compound with wound healing activities.

Materials and Method

Chemical and Drugs

Absolute methanol (Follium Pharmaceuticals, Ethiopia), Ethyl acetate (Sisco research laboratories Pvt. Ltd, India), n-Hexane (Loba Chemie Pvt. Ltd. India), diazepam injection (Gland pharm limited, India), normal saline 0.9% (IV infusion BP Medsol pharmaceuticals), ketamine hydrochloride injection (Neon Laboratories Limited, India). All materials employed were of analytical quality.

Plant Material

V. sinaiticum roots were collected from the Maraki campus, University of Gondar, in Gondar town, Amhara, Ethiopia. The plant material was identified and authenticated by Mr. Abiyu Enyew (botanist at the University of Gondar), and a specimen with voucher number 0002AME/2021 was deposited in the herbarium for future reference.

Extraction and Fractionation

Extraction Powdered roots of *V. sinaiticum* (1kg) were weighed and soaked with 80% methanol (1:6, w/v) at room temperature in Erlenmeyer conical flask covered with aluminum foil.^{15,16} It was then maintained for three days, accompanied by periodic stirring and shaking. After three days, it was filtered through Whatman filter paper (No-1) using pressurized suction filtration. The remaining residue was further macerated with methanol for three days (twice, for a total of six days) to obtain a higher yield. The extract solution was evaporated using a rotary evaporator set at 40°C to remove methanol. Finally, the concentrated aqueous solution was dried by using lyophilizer to remove the aqueous solution. The percentage yield was calculated and stored in a refrigerator at +4°C until use for the formulation of ointments and solvent fractionation.^{16,17}

$$\% \text{ yield} = \frac{\text{weight of extract}}{\text{weight of the plant material}} \times 100$$

Fractionation 80% methanolic crude extract of *V. sinaiticum* roots (85 g) was subjected to different solvents (chloroform, ethyl acetate, and water). The powdered form of the crude extract was suspended in distilled water and shaken to obtain a completely mixed solvent mixture. The mixture was transferred to a separatory funnel and 510 mL of chloroform was added. The mixture was left for some time after shaking until it formed two separate phases, according to their density. The chloroform layer was collected in a separate flask and the procedure was repeated twice by adding an equal amount of chloroform. The aqueous fraction was then fractionated with ethyl acetate (510 mL) three times using a procedure similar to that used for chloroform. Finally, the aqueous residue was obtained. The chloroform and ethyl acetate filtrates were dried at 40°C in an oven and the aqueous fraction was frozen in the refrigerator overnight and dried using a lyophilizer. The percentage yield of each dried fractions was calculated using the formula mentioned in the previous section and stored in a refrigerator at 4°C until it is utilized for ointment formulation as 5% and 10% concentration.^{18,19}

Ointment Preparation

Ointment were prepared based on the formula and proportions stated in the British Pharmacopoeia (Table 1).²⁰

The simple ointment base (100mg) which served as a negative control was prepared by placing hard paraffin in a beaker and melting it in a water bath. The remaining components were added in decreasing order based on their melting points, with cetostearyl alcohol being added first, followed by wool fat and white soft paraffin. The mixture of the ingredients was stirred to obtain a homogenous mixture.

Table I Formula Used for the Preparation of Simple and Medication Ointment

Ingredients	Master Formula (gm)	Reduced Formula (gm)
Wool fat	50 g	5 g
Hard paraffin	50 g	5 g
Cetostearyl alcohol	50 g	5 g
White soft paraffin	850 g	85 g
Total	1000 g	100 g

Crude extracts of 5% and 10% ointments were prepared by mixing 5 g and 10 g of crude extract with 95 g and 90 g of simple ointment, respectively. Whereas, solvent fractions (chloroform, ethyl acetate, and aqueous) were prepared by mixing 1.5 gm (5%) and 3 gm (10%) of each fraction with 28.5 gm and 27 gm of simple ointment respectively. As a negative control, 100 mg of simple ointment without an active ingredient was prepared and nitrofurazone 0.2% was used as a positive control.

Preliminary Phytochemical Screening

The crude extract and solvent fractions (chloroform, ethyl acetate, and water) of the roots of *V. sinaiticum* were screened for the presence of alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins, steroids, tannins, and terpenoid according to standard procedures.²¹

Acute Dermal Toxicity

Dermal toxicity tests were performed according to OECD guidelines 402.²² Adult female mice (20–30 gm, 8–12 weeks) were obtained from animal housing facilities of the Department of Pharmacology, University of Gondar. Six female mice were assigned in two groups, treatment and control.²³ Initially, a preliminary study involved subjecting one mouse to assess the initial dosage. This involved the application of 2000 mg/kg of a ten percent formulation of the hydromethanolic crude extract uniformly over a shaved area, which was then covered with surgical gauze and a nonocclusive bandage for 24 hours. The mouse was individually caged during this exposure period. After 24 hours, any remaining test substance was washed away with water, and no signs of irritation or mortality were observed during this time frame. Subsequently, the remaining two mice from each group were subjected to testing the next day with a limited test dose of 2000 mg/kg of a 10% ointment formulation of the extract. These mice were observed for 24 hours post-application. Observations included immediate scrutiny for any adverse skin reactions after dosing, continuous monitoring for the first half-hour, periodic checks during the initial 24 hours with particular attention in the first four hours, and daily observations for a total of 14 days.^{22,24}

Grouping and Dosing of Experimental Animals

For the excision wound model, four groups each containing six mice were used. Group I mice were treated with a simple ointment, which served as a negative control. Group II and III were treated with 5% and 10% 80% methanol crude extract ointments, respectively. Group IV was treated with 0.2% (w/w) nitrofurazone, which served as the positive control. After observing the effectiveness of the crude extract, the different solvent fractions formulated as ointments were applied as follows. For the solvent fraction, a circular excision wound model and eight groups of mice (six mice in each group) were used. Group I mice were treated with a simple ointment, which served as a negative control. Group II was treated with 0.2% w/w nitrofurazone, which served as the positive control. Groups III and IV were treated with 5% w/w and 10% w/w of the ethyl acetate fraction, respectively, Groups V and VI were treated with 5% w/w and 10% w/w chloroform fractions, respectively, and Groups VII and VIII were treated with 5% w/w and 10% w/w aqueous fractions, respectively.²⁵

For the burn model, grouping was similar to that of excision wound model, whereas for the incision wound model, an additional fifth group was incorporated, which was left untreated group.²⁶

Wound Healing Models

Excision Wound Model

To create an excision wound, ketamine (50 mg/kg) and diazepam (5 mg/kg) were used to anesthetize mice.²⁷ After cleaning with 70% ethanol, the dorsolateral flank was shaved using a surgical blade. A full-thickness circular excision wound measuring approximately 300 mm² was created using toothed forceps, a scalpel, and scissors. The wound was then left undressed in an open environment. The day on which the wound was created was considered day 0. After 24 h, the standard drug, extract, and simple ointment were applied topically once daily, as described in the grouping section.^{28,29}

Measurement of Wound Contraction

The rate of wound contraction was measured using transparent polythene paper and a 1 mm² graph sheet on days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20th post wounding. The wound healing activities of the crude and solvent fractions were calculated using the wound size (300mm²) as 100%, as follows:

$$\% \text{ wound contraction} = \frac{\text{wound area on 0} - \text{wound area on } n}{\text{wound area on 0}} \times 100$$

Where n is the number of days when measurements were taken ie 2nd, 4th, 6th, 8th, 10th, etc. till full wound healing.³⁰

Epithelialization Time Measurement

The number of days required for the scar to fall off from the dead tissue, excluding leaving a raw wound, was taken as the epithelialization period.³⁰

Histopathological Examination

After completion of the experiment, skin specimens were collected from each group of excision and burn wound models. Samples were fixed in 10% buffered formalin, processed, blocked with paraffin, sectioned into 5 µm sections, and stained with hematoxylin and eosin. The sections were examined and recorded by a blinded pathologist and recorded.³¹

Incision Wound Model

In the incision wound model, the mice were anesthetized using the same method as that used for excision, and the dorsal fur of each mouse was shaved. Three cm long and 2 mm depth of incision wound was made using a sterile blade. The wounds were closed with interrupted sutures, 1 cm apart, using a silk no. 00 round and curved needle (no. 11). The wound was left open (Figure 2a).³² After 24 hours of after wound creation, animals were treated as described in the grouping section. The sutures were removed on day 9 and the tensile strength of the healed wound was measured on day 10 using a continuous and constant water flow technique (Figure 2b and c). The anesthetized mice were placed on an operating table. Two forceps were suspended on each side of the edge of the skin, one suspended on the metal rod on the operation table, and the other suspended in a polyethylene bag through a string run over to a pulley. Water was slowly added to the bag until the wound broke. Then the amount of water in grams was recorded and taken as the breaking strength of the wound.^{33,34}

$$\text{Percent tensile strength(TS)of the extract(\%)} = \frac{\text{TS extract} - \text{TS So}}{\text{TS So}} \times 100$$

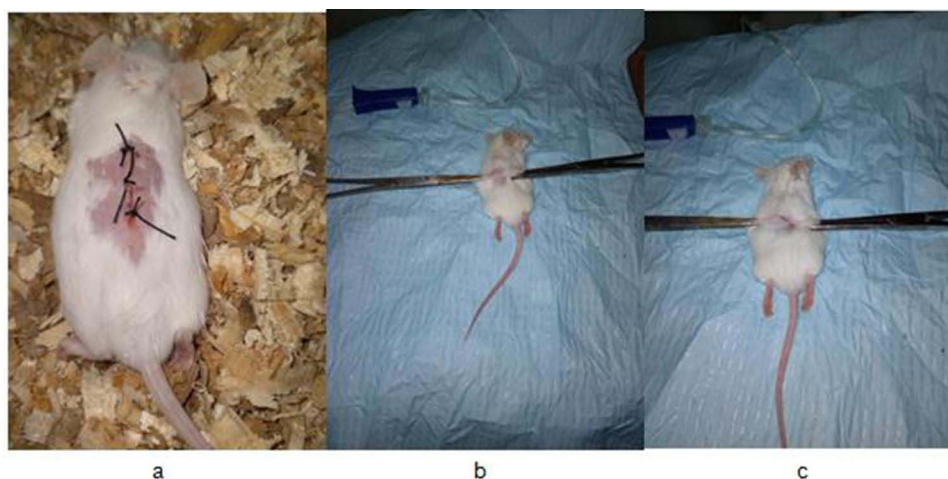


Figure 2 Photograph of incision wound. (a) On the day of wound creation; (b) water flow technique; (c) measuring breaking strength.

$$\text{Percent strength of the reference(\%)} = \frac{\text{TS reference} - \text{TS So}}{\text{TS So}} \times 100$$

$$\text{Percent strength of the simple ointment(\%)} = \frac{\text{TS So} - \text{TS Lu}}{\text{TS So}} \times 100$$

Where So= simple ointment, LU = left untreated²⁸

Burn Wound Model

A partial-thickness burn wound was created by pouring hot molten beeswax at 80°C °C into a metal cylinder with 300 mm² circular openings placed on the shaven area of the mice until the wax solidified. Subsequently, the metal cylinder with wax adhering to the skin was removed. After 24 h, the cells were treated as described in the dosing section. The wound was monitored every two days, and the percentage of wound contraction, epithelialization period, and histopathological analysis were performed.³⁵

Statistical Analysis

All the results are expressed as the mean ± SEM for each group. The differences between groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's tests using SPSS version 26 software, and results were considered significant if P< 0.05.

Results

The Yield of the Extract

The yield of the crude extract was 11%, followed by the ethyl acetate (18.75%), chloroform (12.5%) and aqueous solutions (68.8%).

Acute Dermal Toxicity

During the 24 hour follow-up, dermal toxicity, such as inflammation, swelling, and redness was not observed. The extract did not show any signs of toxicity or mortality during case side observation of 14 days.

Phytochemical Test

Preliminary phytochemical screening of the crude and solvent fractions results is presented in Table 2.

Table 2 Preliminary Phytochemical Screening of the Crude Extract and Solvent Fractions of the Root of *V. Sinaiticum*

Metabolites	Crude Extract	EA Fractions	CF Fractions	AQ Fractions
Anthraquinones	+	+	–	+
Alkaloids	+	+	–	+
Flavonoids	+	+	+	+
Glycosides	–	–	–	–
Tannins	+	–	–	+
Terpenoids	+	+	+	+
Saponins	+	–	–	+
Steroids	–	–	–	–
Phenols	+	+	+	+

Notes: (+) indicates the presence of secondary metabolites, (–) indicates the absence of secondary metabolite.

Abbreviations: CF, Chloroform; EA, Ethyl acetate; AQ, Aqueous.

Table 3 The Effect of the Root *Verbascum Sinaiticum* Crude Extract on Excision Wound Area (mm²) in Swiss Albino Mice

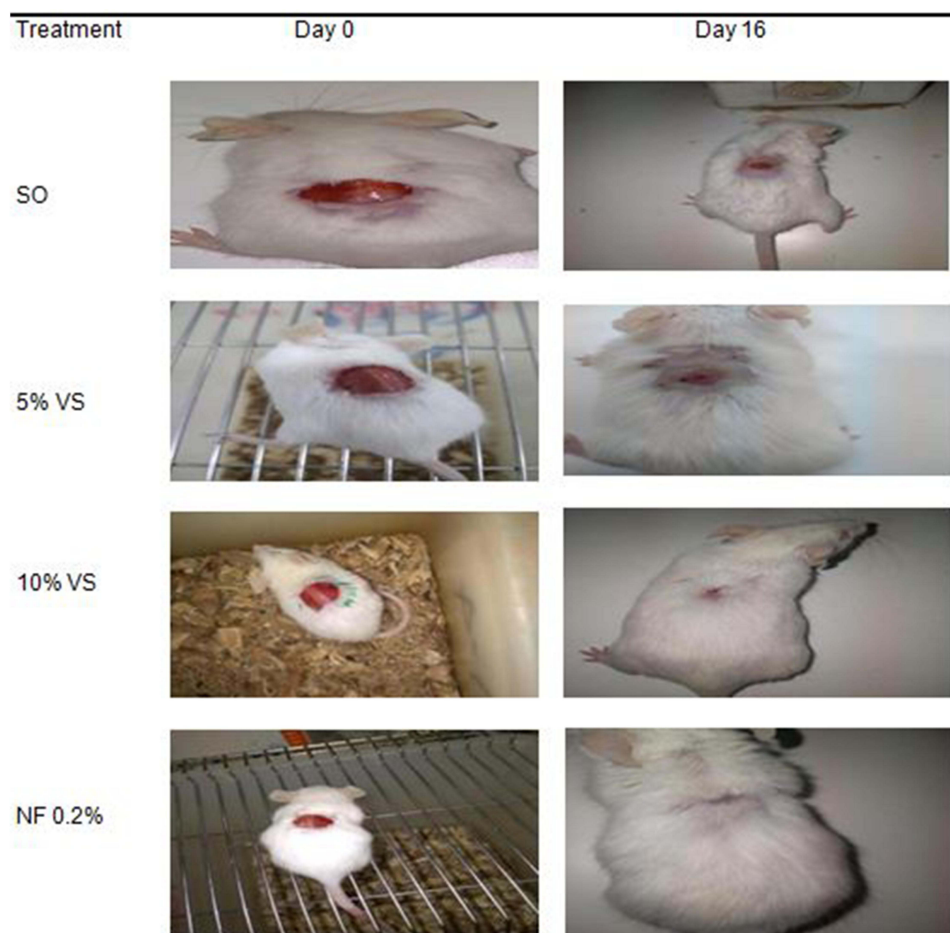
Days	Simple Ointment	5% VS Ointment	10% VS Ointment	NF 0.2%
2	252.5±9.8(15.8%)	231.4±2.9(23%)	231.4±2.9(23%)	231.4±2.9(23%)
4	226.6±5.6(24.5%)	183.1±9.1 (39%)	158.4±10.8(47.2%) ^{a**}	158±10.8(47.2%) ^{a**}
6	188.9±7.7(37%)	145.7±8.7(51.4%) ^{a**}	129.8±7.9(56.7%) ^{a**}	128.1±6.8(57.3%) ^{a**}
8	142.1±9.4(52.6%)	101.3±8.7(66.2%) ^{a**}	84.3±6.1(71.9%) ^{a**}	83.1±7(72.3%) ^{a**}
10	113.5±6.3(62.1%)	74.9±4.3(75%) ^{a**c;d*}	49.8±5.1(83.4%) ^{a**b*}	47.3±7 (84.2%) ^{a**b*}
12	92.7±6.6(69.5%)	51.6±3.7(82.8%) ^{a**c;d*}	31.9±3.2(89.4%) ^{a**b*}	30.7±4.8(89.8%) ^{a**b*}
14	60.3±3.9(79.9%)	26.9±1.9(91%) ^{a**c;d*}	16.6±1.4(94.5%) ^{a**b*}	15.4±1.1(94.9%) ^{a**b*}
16	24.1±2.5(91.9%)	11.2±1.3(96.3%) ^{a**c;d*}	5.3±0.6(98.2%) ^{a**b*}	4.7±0.6 (98.4%) ^{a**b*}
18	9.9±1.6(96.7%)	3.8±0.6(98.7%) ^{a**c;d*}	0.0±0.0(100%) ^{a**b*}	0.0±0.0(100%) ^{a**b*}
EPD	20.2±0.4	16.2±0.7 ^{a**}	15.3±0.4 ^{a**}	15±0.9 ^{a**}

Notes: Data are expressed as Mean± SEM; n=6 and analyzed by one way ANOVA followed by post hoc Tukey's test; values in parenthesis express percent of wound contraction; a=compared to simple ointment; b=compared to 5% VS; c=compared to 10% VS; d=compared to NF 0.2%; *=P<0.05; **=P<0.001.

Abbreviations: EPD, Epithelialization Day; VS, *Verbascum sinaiticum*; NF, Nitrofurazone.

Effect of *Verbascum Sinaiticum* Root on Wound Healing Excision Wound Model

The results obtained from the excision wound model revealed that the 5% and 10% crude extracts showed a significant wound healing effect compared to the simple ointment (Table 3, Figure 3).

**Figure 3** Photograph of excision wound mice treated with simple ointment, 5% crude extract, 10% crude extract and nitrofurazone 0.2%.

Abbreviations: SO, Simple Ointment; VS, *Verbascum Sinaiticum*; NF, Nitrofurazone.

No significant difference was observed in the percentage of wound contraction until day 4 between the simple ointment-treated groups and groups treated with 5% VS, 10% VS, and the standard drug (NF 0.2%). From day 4 to day 18, the 10% crude extract-treated group showed significant ($P<0.001$) wound contraction compared to the simple ointment-treated group. In comparison with the 5% crude extract-treated group, the 10% vs NF 0.2% treated groups appeared to have statistically significant ($P<0.05$) percentages of wound contraction from day 10 to day 18 and the maximum percentage of wound contraction (98.2% and 100%) in the 10% crude extract-treated group was observed on day 16 and day 18 respectively. However, no significant difference was observed in wound contraction between the 10% VS and NF 0.2% treated groups. In contrast, the 5% VS- treated groups showed a significant percentage of contraction ($P<0.001$) starting from day 6 onwards in comparison with that of simple ointment-treated groups and the maximum percentage of wound contraction (98.7%) among the 5% VS-treated groups was observed on day 18.

In terms of shortening of the epithelialization period, 5% VS (16.2 days), 10% VS (15.33 day), and NF 0.2% (15 days)-treated groups showed a significant ($P<0.001$) effect compared to the simple ointment (20.20 day)-treated groups in improving the rate of epithelialization.

Histopathological Analysis

The 10% crude extract formulation-and 0.2% nitrofurazone-treated mice showed moderate fibroblast concentration, collagen deposition, and the absence of inflammatory cells (Figure 4c and d). Likewise, mice treated with the 5% crude extract showed low concentrations of inflammatory cells, moderate fibroblast concentrations, and neovascularization (Figure 4b). The simple ointment-treated groups showed moderate inflammatory cells, an absence of fibroblast proliferation, and collagen deposition (Figure 4a).

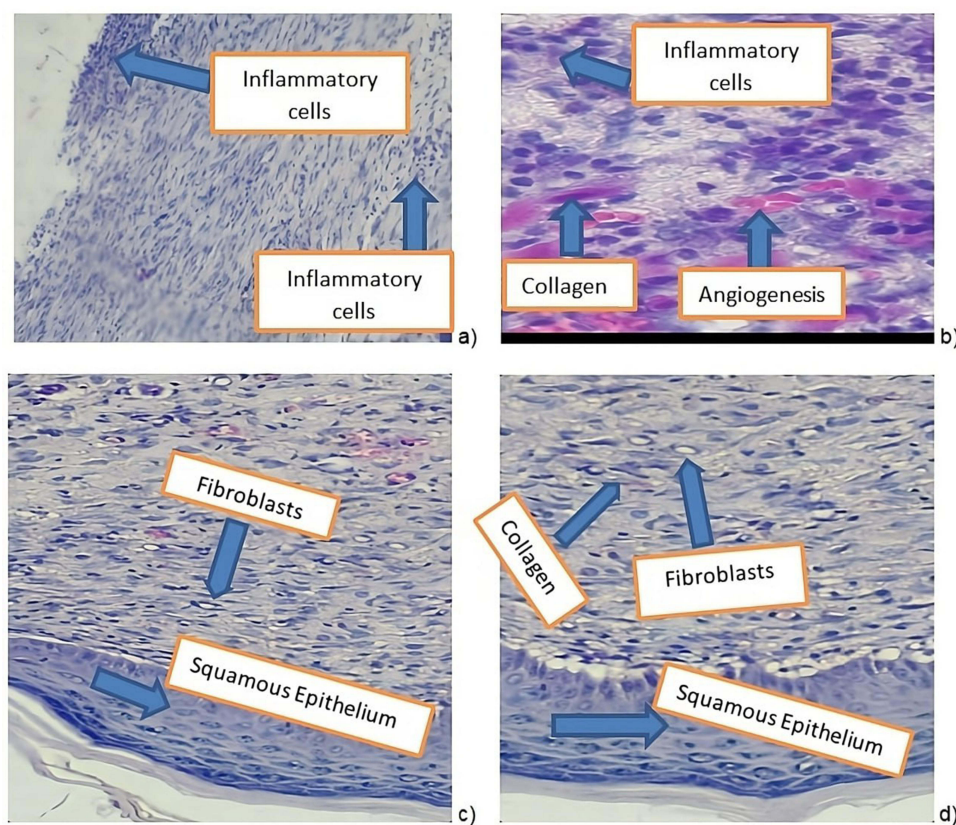


Figure 4 Photograph of the histological specimen of excision wound tissue. (a) Histological section of simple ointment; (b) Histological section of 5% crude extract; (c) Histological section of 10% crude extract; (d) Histological section of Nitrofurazone.

Incision Wound Model

The effects of *V. sinaiticum* root extract on the incision wound are shown in Table 4 and Figure 2. The mean tensile strength of the groups treated with 10% VS and NF 0.2% was found to be significant ($P<0.001$) in increasing the breaking strength by 60.5% and 62.5%, respectively, compared to the untreated and simple ointment-treated groups. In addition, significant effects ($P<0.05$) on breaking strength were observed in groups treated with 10% VS and NF 0.2%, when compared to the 5% VS, treated group. In contrast, groups treated with 5% crude extract showed a significant ($P<0.001$, $P<0.01$) increase in breaking strength compared to the untreated and simple ointment treated groups, respectively.

Burn Wound Model

The root of *Verbascum sinaiticum* root extract showed a significant effect on wound contraction and the period of epithelialization on alternate days of burn wound treatment (Table 5 and Figure 5). Groups treated with 10% VS and NF 0.2% showed a significant ($P<0.001$) percentage of wound contraction starting from day 4 onwards compared to the simple ointment treated group. Compared to the 5% crude extract-treated group, the 10% crude extract-and 0.2% nitrofurazone-treated groups showed significant ($P<0.01$) wound contraction from days 12 to 18. In contrast, groups treated with 5% ointment showed a significant ($P<0.001$) effect from day 8 onwards on wound contraction compared to groups treated with simple ointment.

Table 4 Effect of Crude Extract of the Root of *Verbascum Sinaiticum* Extract on Tensile Strength in an Incision Wound Model

Group	Breaking Strength in Gram (Mean±SEM)	Percent tensile Strength
Left untreated	246.1±7.3	—
Simple ointment	266±6.4	8.1%
5% VS	400.7±4.2 ^{a***b**}	50.6%
10% VS	426.9±4.4 ^{a***b***c*}	60.5%
NF 0.2%	432.2±5.8 ^{a***b***c*}	62.5%

Notes: Data are expressed in Mean ± SEM; n=6 and analyzed by one way ANOVA followed by post hoc Tukey's test; a=compared to simple ointment; b =compared to 5% crude extract; c=compared to 10% crude extract; *= $P<0.01$; **= $P<0.01$; ***= $P<0.001$.

Abbreviations: VS, *Verbascum sinaiticum*; NF, Nitrofurazone.

Table 5 Effect of the Crude Extract of the Root of *Verbascum Sinaiticum* Extract on Burn Wound Model in Swiss Albino Mice

Days	Simple Ointment	5% VS Ointment	10% VS Ointment	NF 0.2%
2	249.2±5.8(16.9%)	240±6.1(20%)	231.5±4.7(22.8%)	226.7±0.0(24.4%)
4	231.4±9.7(22.9%)	201.1±9.6(32.9)	173.1±7.2(42.3%) ^{a**}	169.3±7.8(43.6%) ^{a**}
6	209.5±9.2(30.2%)	169.5±9.6(43.5%)	123.4±8.4(58.9%) ^{a**}	116.8±8(61.05%) ^{a**}
8	177.2±8.6(40.9%)	123.9±11(58.7%) ^{a**}	89.9±7(70.02%) ^{a**}	84.7±8.1(71.8%) ^{a**}
10	136.2±7.5(54.6%)	79.9±4.5(73.4%) ^{a**}	46.8±4.9(84.4%) ^{a**}	41.4±5.5(86.3%) ^{a**}
12	101.1±5.4(66.3%)	50.4±2.9(83.2%) ^{a***c*d*}	20.4±3.9(93.2%) ^{a***b*}	16.4±2.7(94.6%) ^{a***b*}
14	68.7±4.4(77.1%)	26±3.6(91.3%) ^{a***c*d*}	4.5±0.8(98.5%) ^{a***b*}	3.1±0.0(98.9%) ^{a***b*}
16	31.3±2.9(89.5%)	11.6±0.9(96.1%) ^{a***c*d*}	2.9±0.7(99.3%) ^{a***b*}	2.1±0.7(99.3%) ^{a***b*}
18	20.2±2.4(93.3%)	4.4±0.8(98.5%) ^{a***c*d*}	1.5±0.2(99.6%) ^{a***b*}	0.0±0.0(100%) ^{a***b*}
20	12.8±1.6(95.7%)	1.7±0.6(99.4%) ^{a**}	0.0±0.0(100%) ^{a*}	0.0±0.0(100%) ^{a**}
EPD	19.3±0.5	15.7±0.6 ^{a**}	14.2±0.5 ^{a**}	14±0.7 ^{a**}

Notes: Data are expressed in Mean ± SEM; n=6 and analyzed by one way ANOVA followed by post hoc Tukey's test; a=compared to simple ointment; b =compared to 5% crude extract; c=compared to 10% crude extract; d= compared to NF 0.2%; *= $P<0.01$; **= $P<0.001$.

Abbreviations: EPD, Epithelialization days; VS, *Verbascum Sinaiticum*; NF, Nitrofurazone.

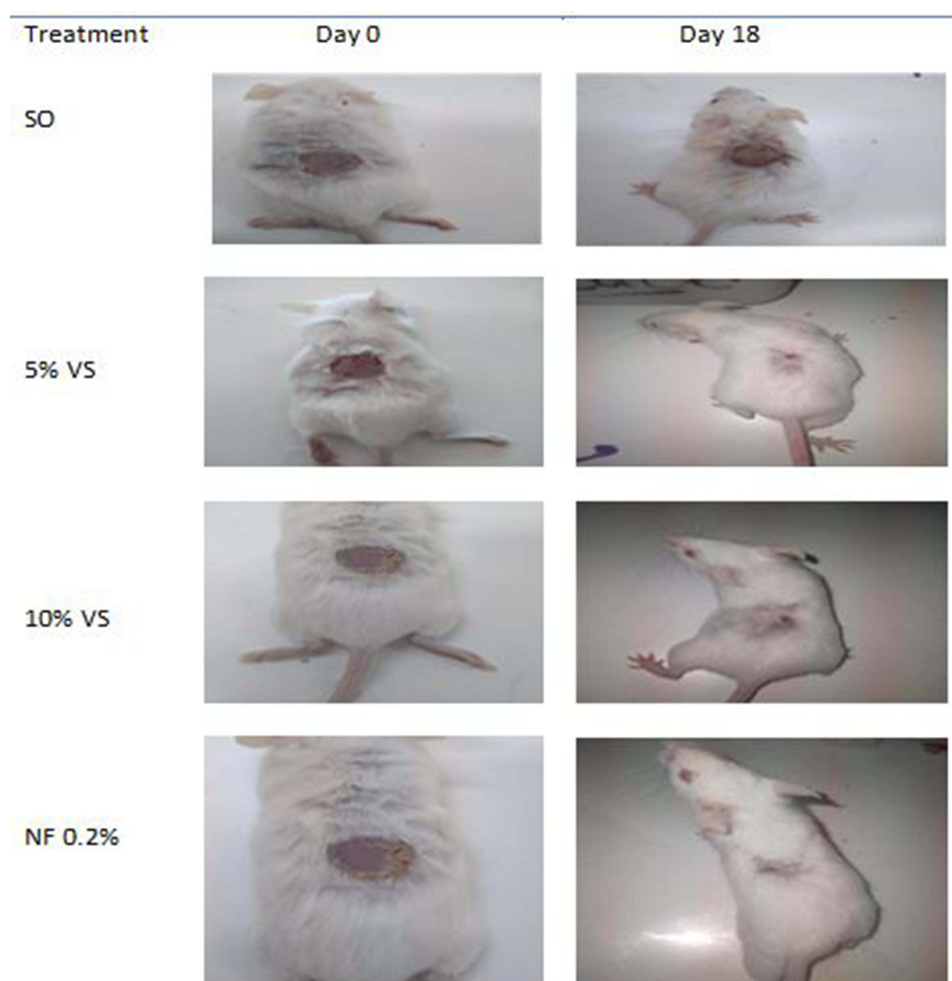


Figure 5 Photograph of burn wound in mice treated with simple ointment, 5% VS crude extract, 10% VS crude extract, and nitrofurazone 0.2%.
Abbreviations: SO, Simple Ointment; VS, *Verbascum Sinaiticum*; NF, Nitrofurazone.

At the time of shortening of the epithelialization period, the 5% VS, 10% VS, and NF 0.2% treated groups showed comparable and minimal days compared to the simple ointment-treated groups. Significant effects on the rate of epithelialization were observed in groups treated with 5% VS (15.7 days, $P < 0.001$), 10% VS (14.2 days, $P < 0.001$), and NF 0.2% (14 days, $P < 0.001$) compared to the simple ointment treatment group.

Histopathological Analysis

Skin specimens from the burn model mice were taken on the 20th day of treatment. The highest dose of the extract (10%) and nitrofurazone (0.2%) resulted in a higher concentration of fibroblasts, collagen deposition, and a lower concentration of inflammatory cells. Mice treated with 5% crude extract also showed lower concentrations of inflammatory cells, moderate fibroblasts, neovascularization, and collagen deposition. In contrast, mice treated with a simple ointment showed a high concentration of inflammatory cells, low concentration of fibroblast proliferation, and an absence of collagen deposition.

Wound Healing Effects of the Solvent Fraction of *Verbascum Sinaiticum* Root on Excision Wound

The effects of chloroform, ethyl acetate, and the aqueous solvent fraction of *V. sinaiticum* root on the area of the excision wound and the period of epithelialization were found to be significant at different p-values. However, the groups treated with both formulations of the chloroform fraction failed to show any significant effect compared to the simple ointment-treated group (Table 6). Groups treated with the ethyl acetate 5% fraction showed significant wound contraction ($P < 0.01$) on day 6 and ($P < 0.001$) from day 8 to day 18 as compared to the simple ointment treated group. In contrast, the 5% ethyl acetate-treated groups

Table 6 Effect of Solvent Fractions of *Verbascum Sinaiticum* Root on Excision Wound Area in Swiss Albino Mice

Group	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18
SO	258±9 (14%)	229.6±5 (23.5%)	190.9±7 (36.4%)	148.1±9 (50.6%)	115.5±8 (62%)	94.7±5.7 (68.4%)	65.3±4.9 (78.2%)	25.1±3 (91.6%)	10±1.9 (96.7%)
CF 5%	245.2±5 (18.3%)	227.1±6.9 (24.3%)	189.3±10 (36.9%)	150.6±6 (49.8%)	133.1±7 (55.6%)	90.4±9.2 (69.9%)	66.5±5.7 (77.8%)	32±3.9 (89.3%)	18.7±2.4 (93.8%)
CF 10%	240.6±6 (19.9%)	218.2±5.5 (27.3%)	177.6±11 (40.8%)	151.5±10 (49.5%)	129.7±6 (56.7%)	89.8±5.7 (70.1%)	62.6±7.1 (79.1%)	32±3.2 (89.3%)	17.5±2.6 (94.2%)
EA 5%	236.1±5 (21.3%)	201.2±6.5 (34.2%)	155.4±13 (48.2%) ^{a*}	101.3±5 (59.9%) ^{a***b***c***}	66.5±5.7 (71.8%) ^{a***b***c***}	42.7±3.7 (81.6%) ^{a***b***c***}	25.6±3.1 (91.5%) ^{a***b***c***}	11±2 (96.3%) ^{a***b***c***}	5.1±0.9 (98.3%) ^{a***b***c***}
EA 10%	232±4.6 (22.9%)	184.9±8.4 (38.4%) ^{a**}	133.3±9 (55.6%) ^{a***b***c***}	95.8±8.1 (68.1%) ^{a***b***c***}	64.2±6.7 (78.6%) ^{a***b***c***}	37±3.4 (87.7%) ^{a***b***c***}	18.7±2.4 (93.8%) ^{a***b***c***}	10.8±1.2 (96.4%) ^{a***b***c***}	4.5±0.8 (98.5%) ^{a***b***c***}
AQ 5%	231.4±6 (24.3%)	181.5±11 (39.5%) ^{a**}	130.2±10 (56.6%) ^{a***b***c***}	92.5±5.3 (69.2%) ^{a***b***c***}	59.3±4.6 (80.2%) ^{a***b***c***}	35.3±3.6 (88.2%) ^{a***b***c***}	17.5±2.6 (94.2%) ^{a***b***c***}	8.2±1.5 (97.3%) ^{a***b***c***}	2.6±0.5 (99.1%) ^{a***b***c***}
AQ 10%	222.5±4 (25.8%)	165.2±5.1 (44.9%) ^{a***b***c***}	110±3 (59%) ^{a***b***c***}	86.7±3.7 (71.1%) ^{a***b***c***}	52.6±3.8 (82.5%) ^{a***b***c***}	23±3.7 (92.3%) ^{a***b***c***}	9.4±2.4 (96.9%) ^{a***b***c***}	2.1±0.7 (99.3%) ^{a***b***c***}	0.0±0.0 (100%) ^{a***b***c***}
NF 0.2%	221.9±2 (26%)	155.2±9 (48.2%) ^{a***b***c***}	108.1±6 (63.9%) ^{a***b***c***}	81.4±6.9 (72.9%) ^{a***b***c***}	49.6±4.5 (83.5%) ^{a***b***c***}	22.9±2.9 (92.3%) ^{a***b***c***}	9.2±1.6 (96.9%) ^{a***b***c***}	1.9±0.7 (99.4%) ^{a***b***c***}	0.0±0.0 (100%) ^{a***b***c***}

Notes: Data are expressed in Mean ± SEM; n=6 and analyzed by one way ANOVA followed by post hoc Tukey's test; a= compared to simple ointment; b=As compared to chloroform 5%; c= As compared to chloroform 10%; *= P<0.05; **= P<0.01; ***= P<0.001.

Abbreviations: SO, Simple Ointment; CF, Chloroform; EA, Ethyl acetate; AQ, Aqueous; NF, Nitrofurazone.

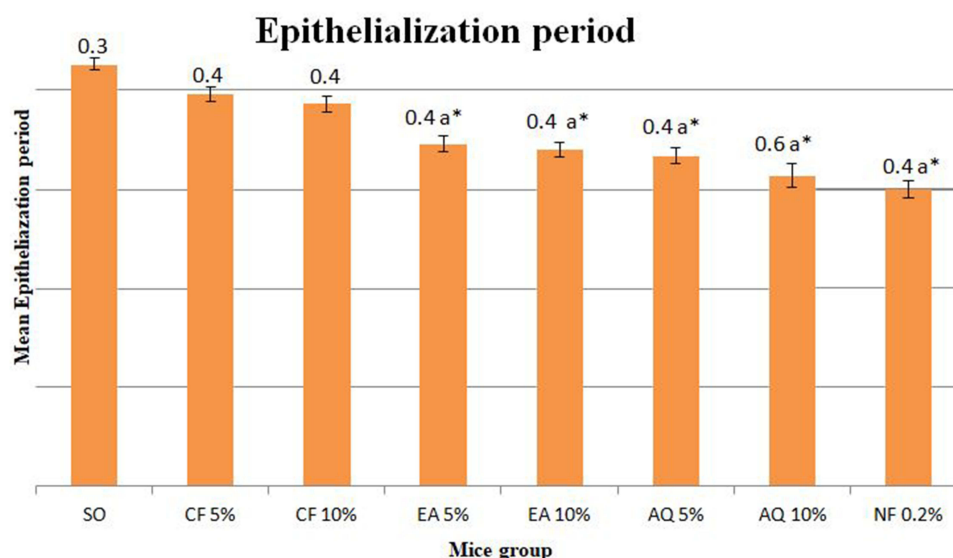


Figure 6 Effect of solvent fractions of *Verbascum sinaiticum* on epithelialization period. a, compared to simple ointment; *= $P<0.001$.
Abbreviations: SO, Simple ointment; CF, Chloroform; EA, Ethyl acetate; AQ, Aqueous; NF, Nitrofurazone 0.2%.

showed significant wound contraction ($P<0.001$) from day 8 onwards compared to the 5% and 10% chloroform-treated groups. The ethyl acetate 10% and aqueous 5% fraction-treated groups showed significant wound contraction ($P<0.01$) beginning on the 4th day and ($P<0.001$) on the 6th day onwards compared to the simple ointment-treated group. Both fraction-treated groups showed a higher percentage of wound contraction ($P<0.01$) on day 6 and ($P<0.001$) from day 8 onwards than the 5% and 10% chloroform fraction-treated groups.

On the other hand, groups treated with aqueous 10% and nitrofurazone 0.2% showed a significant percentage of contraction ($P<0.001$) starting from the 4th day onwards when compared to the simple ointment, 5% chloroform, and 10% chloroform fraction-treated groups.

Shortening of the epithelialization period was observed at a significant level in groups treated with aqueous (5% VS and 10% VS) and ethyl acetate (5% VS and 10% VS) fractions of *V. sinaiticum* roots compared to groups treated with simple ointment (Figure 6). Groups treated with ethyl acetate (5% and 10%), aqueous (5% and 10%), and nitrofurazone 0.2% showed a significant effect ($P<0.001$) on the shortening of the epithelialization period compared to the simple ointment-treated group. Among these groups, mice treated with aqueous 10% and 0.2% nitrofurazone showed the shortest number of days. However, groups treated with 5% and 10% chloroform failed to show any significant effect on the number of days of shortening compared to the simple ointment-treated groups (Figure 6).

Discussion

Verbascum sinaiticum, a well-known plant in Ethiopia, is used by traditional healers for wound management. In different ethnobotanical studies, the powdered root of *V. sinaiticum* was topically applied to the affected area^{10–12} by mixing with butter.¹⁰ In this study, powdered roots were extracted. It was prepared in a simple ointment base that assimilated traditional use.³⁶

No irritation or toxicity was observed during the dermal toxicity test, which confirmed that the methanolic crude extract of *V. sinaiticum* root is safe for use in topical preparation.

In a previous study on the roots of *V. sinaiticum*, different solvent fractions of the root extract showed the possible presence of secondary metabolites.³⁷ Similarly, the current findings show that qualitative phytochemical screening of the crude extract and solvent fraction showed the possible presence of anthraquinone, alkaloids, flavonoids, phenols, tannins, saponins, and terpenoids.

Phenolic compounds and flavonoids are known to have antioxidant activity with considerable free radical scavenging activities.^{38,39} Tannins have a significant impact on the wound healing because of their antibacterial, antioxidant, and

anti-inflammatory activities.⁴⁰ Anthraquinone plays a role in wound healing by reducing inflammation and promoting tissue regeneration by enhancing the expression and signaling of growth factors.⁴¹ Therefore, secondary metabolites may partly contribute to the observed healing of wounds, alone or in combination, in the three models used in this study.

In the three models in this study, the 10% crude extract showed better wound healing activity than the 5% crude extract, which may be due to the concentration of the bioactive constituents, which may increase as the dose increases.

In the excision wound model, the wound healing effect of the extract in both formulations (5% w/w and 10% w/w) resulted in fast wound contraction and increased in the rate of epithelialization. This effect is probably similar to that of a previously studied plant in the same genus (*V. speciosum*) which showed fast wound contraction and resulted in shortening of the epithelialization period.^{42,43} The observed wound-healing effects of the crude extract in mice might be due to its antibacterial and antioxidant activities.

The crude extract of the root of *V. sinaiticum* and its solvent fractions in a previous study showed a significant inhibitory effect on *S. aureus*, *S. epidermidis*, *K. pneumonia*, and *E. coli*.^{11,37} These pathogens colonize wounds and delay healing by releasing toxins.⁴⁴ The antibacterial activity is consistent with a previous study conducted on *Verbascum thapsus* methanolic extract, which possesses antibacterial activity against common pathogens mentioned above⁴⁵ and the antioxidant activity is probably similar to the study performed in the same genus of three *Verbascum* species whose methanolic extracts showed good antioxidant activity.⁴⁶

In addition, the healing effect of the crude extract on excision wounds might be due to an increase in fibroblast proliferation and collagen deposition, as evidenced by histopathology analysis.

Furthermore, the wound healing activity of 80% methanolic crude extract was observed in the incision wound model by measuring the tensile strength. The tensile strength of the wound mainly relies on the synthesis of collagen and its maturation,⁴⁷ and the greater the deposition of collagen, the stronger the wound becomes in resisting breaking.⁴⁸ Thus, the observed effect on tensile strength might be due to the ability of the extract to increase the rate of synthesis and maturation of collagen.⁴⁹ The increase in tensile strength may be similar to a study done on the same genus (seven *Verbascum* species) that revealed an increase in tensile strength, which might be associated with the presence of fibroblast proliferation and collagen deposition, as evidenced by histopathological analysis.⁵⁰

In contrast, topical application of 5% crude extract and 10% crude extract ointment on burn wounds showed significant wound healing compared to simple ointments. Burn wounds are known to be associated with high inflammation, which allows matrix metalloproteinase (MMP) and other enzymes to destroy the extracellular matrix and render the site of the wound vulnerable to infection.^{51,52} This destruction as a result of inflammation can be prevented or destroyed by medicinal plants via reduction of excessive inflammation.⁵³

According to a previous study done on *Verbascum* species, the species of *Verbascum* are known to possess anti-inflammatory activity, and it was indicated to be the principal mechanism for their wound healing activity⁵⁴ which might indicate the wound healing effect of the root of *V. sinaiticum* is a result of its anti-inflammatory activity. An additional study conducted in the same genus (*Verbascum pterocalycinum*) also reported anti-inflammatory and antinociceptive activities⁵⁵ which probably similar in effect with the root of *V. sinaiticum*.

The crude extract in the three models of the study with 5% and 10% concentration showed a decrease in the activity of wound healing as compared to the positive control, nitrofurazone 0.2%. The crude extract can exhibit significant variation in potency and may encompass inconsistent levels of bioactive compounds. The concentration of active compounds within the crude extract fall below a certain threshold, its wound healing activity might not match that of nitrofurazone.

The crude extract was further fractionated with different solvents, and according to the results of this study, the aqueous and ethyl acetate fractions showed significant wound healing activity compared with the simple ointment-treated groups.

In a previous study on the root of *V. sinaiticum*, it was reported that the highest yield was obtained in a polar solvent, and which implies that the roots of *V. sinaiticum* seem to be polar, which is consistent with the results of the current study.³⁷

The difference in wound-healing activity among the solvent fractions might be due to their ability in inhibiting bacterial growth and the presence and effect of secondary metabolites. This idea was supported by two previously

reported in vitro studies on the root of *V. sinaiticum* crude extract and the solvent fraction.^{11,37} According to those studies, the aqueous and chloroform fractions exhibit broad-spectrum antibacterial activity. The result is in line with the current finding that higher activity of the aqueous fraction of *V. sinaiticum* might be a result of broad-spectrum antibacterial activity.

Nevertheless, the chloroform fraction did not exhibit any notable impact on wound contraction or epithelialization duration compared to the simple ointment. This outcome could stem from the chloroform fraction's inability to effectively release its active ingredient in vivo, and/or the insufficient concentration of secondary metabolites within the chloroform fraction, which may not provide adequate antibacterial and antioxidant activity in vivo.

When the fractions compared to crude extract excision wound model, the fractions showed a decrease wound healing effect. The reduced wound-healing effect observed in the fractions compared to the crude extract may be the crude extract may contain a combination of active compounds that work synergistically to promote wound healing. When the extract is fractionated, some of these synergistic interactions may be lost, leading to reduced activity of wound healing effect in the fractions. In addition, fractionation of the crude extract may result in the removal of certain bioactive compounds that are responsible for the wound-healing effect. These compounds may work in concert with others in the crude extract to produce the observed therapeutic effect.

In addition, the fractions of the crude extract showed a decrease in the wound healing effect than the standard drug, nitrofurazone 0.2%. Fractions may lack synergistic effects among the compounds present in the crude extract, which are necessary for optimal wound healing. Nitrofurazone, as a single compound, may possess synergistic properties that enhance its healing effect.

The crude extract and its fractions demonstrated wound healing effects across all three wound models. These effects are likely attributed to the antibacterial, anti-inflammatory, and antioxidant properties associated with the medicinal activity of the *Verbascum* genus, as supported by relevant studies.^{54,56,57}

Limitations

Owing to the unavailability of operative machines that count the number of specific cells in the specimen, the concentration of cells in the specimen was subjectively ranked by a pathologist. This makes the results obtained from the histopathological analysis less accurate and tangible.

Conclusion

The root extracts of the 80% methanolic crude extract, aqueous fraction, and ethyl acetate fraction of *V. sinaiticum* showed significant wound healing activity in the models used in this study. However, the chloroform fraction of *V. sinaiticum* failed to show any significant wound healing activity. The observed wound healing effect might be associated with the presence of different phytochemical constituents in the extract. This justifies the use of *V. sinaiticum* root as a wound healing agent.

Data Sharing Statement

The corresponding author can provide the datasets utilized or examined in this study upon reasonable request.

Ethics Approval

The experiment was conducted in accordance with the guidelines for handling and care.⁵⁸ The study commenced after ethical clearance was obtained from Department of Pharmacology, College of Medicine and Health Sciences, University of Gondar (protocol number SOP4/97/13). Experiments were carried out, and the data were compiled in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, National Research Council (2012).

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Author Contributions

All authors made a significant contribution to the work reported, in the conception, study design, execution, acquisition of data, analysis and interpretation, and also; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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