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# Evaluation of wound healing and anti-inflammatory activity of the leaves of *Calpurnia aurea* (Ait.) Benth (fabaceae) in mice

Getachew Ayal[a,*], Assefa Belay[b], Wubayehu Kahaliw[b]

[a] Debre Markos Referral Hospital, Debre Markos, Ethiopia
[b] Department of Pharmacology, School of Pharmacy, University of Gondar, Gondar, Ethiopia

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ABSTRACT

*Ethnopharmacological relevance:* The leaves of *Calpurnia aurea* (Ait.) Benth are used for wound healing activities through topical application in different communities in Ethiopia. The leaves had many confirmed in vitro activities that can promote wound healing effects. In spite of many claims and in vitro studies with supportive results in wound healing, no scientific study has been conducted on the wound healing and anti-inflammatory activity of leaves of *Calpurnia aurea* (Ait.) Benth on animals.

*Aim of the study:* was to evaluate the wound healing and anti-inflammatory activity of leaves of *Calpurnia aurea* (Ait.) Benth scientifically in mice.

*Methods and materials:* Air dried leaves of *Calpurnia aurea* (Ait.) Benth were grounded and macerated three times successively by 80% methanol. Then part of the dried extract was fractionated with chloroform, ethyl acetate and aqueous. Phytochemical screening tests were performed according standard test procedures. The crude extract and solvent fractions were formulated to ointments. The acute dermal toxicity of the crude extract was determined in rats. Wound healing activity of the crude extract was evaluated using excision and incision wound models and the wound healing activities of solvent fractions were evaluated by using excision wound model. The anti-inflammatory activity of the 80% methanol extract of leaves of *Calpurnia aurea* (Ait.) Benth was evaluated using carrageenan induced hind paw edema model in mice.

*Results:* The 2000 mg/kg test dose of the 10% w/w crude extract ointment was safe in rats. The 5% w/w and 10% w/w crude extract ointments revealed significant ($p < 0.001$) wound contraction from day 4-day 8 post wounding. The 10% w/w crude extract ointment revealed a significant ($p < 0.01$) shortening of epithelialization period in excision wound model. Both 5% w/w and 10% w/w crude extract ointments showed significant ($p < 0.001$) increment of tensile strength compared to the negative control. The 10% w/w aqueous fraction ointment revealed high ($p < 0.001$) percentage of wound contraction and reduced ($p < 0.001$) period of epithelialization. The 100 mg/kg, 200 mg/kg and 400 mg/kg oral administration of the crude extract had significant inhibition of the paw edema in mice of carrageenan induced inflammation.

*Conclusion:* The results of this study demonstrated that 80% methanol extract of the leaves of *Calpurnia aurea* (Ait.) Benth exhibited wound healing and anti-inflammatory effects. The aqueous and ethyl acetate fractions possessed wound healing activities.

## 1. Introduction

Wound is defined as the disruption of the anatomic and cellular integrity of tissue due to chemical, physical, thermal, microbial, or immunological insult [1] thereby causing a perturbation in the normal skin anatomy and function [2]. Depending on the underlying cause of creation, wounds can be either closed or open wounds [3,4]. In open wounds, the skin is cracked open and the underlying tissue is exposed to the outside environment making it more vulnerable to bleeding and infection. But, in closed wounds the skin is intact and the underlying tissue is not directly exposed to the outside environment [4]. Based on time of healing, wounds can be either acute or chronic wound [5]. Acute wounds are typically due to some form of trauma that could be blunt or penetrating [6] and repair themselves through proceeding timely and orderly healing pathway, with the end result of both functional and anatomical restoration within 30 days [5]. Chronic wounds are those that could not proceed through an orderly and timely reparative process to produce anatomic and functional integrity of the

* Corresponding author.
*E-mail address:* getayal15@gmail.com (G. Ayal).

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**Fig. 1.** Calpurnia aurea (Ait.) Benth from site of collection (Captured on 20/12/2017).

injured site [7]. Wound healing is the body's natural process to regenerate dermal and epidermal tissue [8] through a coordinated physiological response which provides hemostasis and initiates the processes of inflammation, proliferation, and remodeling [6].

*Calpurnia aurea* (Ait.) Benth (Fig. 1) is a yellow-flowered small tree or shrub alternatively called Natal Laburnum [9]. *Calpurnia aurea*(Ait.) Benth is known by several local names in Ethiopia, digita (ደግጣ) in Amharic and chekata in Afaan Oromo [10]. In Ethiopia, *Calpurnia aurea* (Ait.) Benth is traditionally used as anti-scabies [11], anti-malarial [12] and anti-diarrheal [13] herb. It is used to treat swelling, tuberculosis [14], rabies [15], excessive menstruation and fungal disease on the skin [16], amoebiasis, giardiasis, diabetes and hypertension [17]. The leaves of *Calpurnia aurea* (Ait.) Benth are used for wound healing activities through topical application in communities around Dek islands, Gozamin district and Fiche [15,18,19]. The Hadya people use *Calpurnia aurea* (Ait.) Benth to treat skin diseases [20].

In spite of many claims and in vitro studies with supportive results in wound healing, no scientific study has been conducted on the wound healing and anti-inflammatory activity of leaves of *Calpurnia aurea* (Ait.) Benth on animals. An earlier study recommends evaluating the wound healing and anti-inflammatory activity of leaves of *Calpurnia aurea* (Ait.) Benth [21]. Hence it was essential to evaluate the wound healing and anti-inflammatory activity of leaves of *Calpurnia aurea* (Ait.) Benth as the leaves may be a possible source of effective, safe and affordable wound healing agents.

## 2. Materials and methods

### 2.1. Drugs and chemicals

The drugs, chemicals and reagents used in this study were of the required standard and analytical grade. They were purchased from local suppliers. These include nitrofurazone ointment USP 0.2% (shanghai general pharmaceutical CO., LTD, China), ketamine hydrochloride injection USP (Neon Laboratories limited, India), diazepam injection (Gland pharm limited, India), halothane inhalation (Piramal enterprises limited, India), hard paraffin (Lab tech chemicals), wool fat (UNI-CHEM chemical reagents), white soft paraffin (Ethiopian pharmaceuticals manufacturing, SH.CO), Cetostearyl alcohol (Blulux Laboratories (P) Ltd., India), carrageenan, Tween 80 (UNI-CHEM Chemical Reagents, India), distilled water (UOG, Pharmaceutics laboratory), indomethacin (Leben Laboratories Industries PVT, LTD, India), methanol absolute (AppliChem, Germany), ethyl acetate (Carlo Erba reagent S.A.S., France), chloroform (Nice Chemicals Pvt, India), sodium hydroxide (supertek chemicals), Wagner's reagent (Research-Lab Fine Chem Industries, India), lead acetate (Guangdong Guanghua chemicals factories, China), sulphuric acid (HiMedia Laboratories Pvt. Ltd., India) and acetic anhydride.

### 2.2. Instruments, apparatus and supplies

Mortar and pestle, rotary evaporator, digital weighing balance, lyophilizer, deep freezer, ointment slab, Whatman filter paper (Number 1), Plethysmometer (Orchid scientific, PLM-01-Plus), sharp sterilized scissors, surgical threads with curved needles, forceps, permanent marker and graph paper were used in this study.

### 2.3. Plant material

Fresh leaves of *Calpurnia aurea* (Ait.) Benth were collected from Gozamin district, East Gojjam Zone, Amhara Region, North West of Addis Ababa on December 2017. The plant was authenticated by Botanists in Department of Biology, College of Natural and Computational Sciences, University of Gondar where a specimen was deposited for future reference with voucher number GA008/2010.

### 2.4. Extraction and fractionation

The leaves of *Calpurnia aurea* (Ait.) Benth were washed under running water to remove surface pollutants and air dried under shade at room temperature. Then, the dried leaves were coarsely powdered using a mortar and pestle. Cold maceration extraction technique was used to extract the plant material [22]. One kilo gram powder was macerated with 5 litter 80% methanol for three days in an Erlenmeyer conical flask with occasional stirring. Then, the extract was first filtered using gauze and then with Whatman filters paper No. 1. The residue was re-macerated twice in the same manner to maximize the yield. The filtrates were combined and dried by using rotary evaporator at 40 °C. The filtrates were further concentrated using dry oven at 40 °C. Then the concentrated extract was frozen overnight using deep freezer followed by drying with a lyophilizer at −50 °C and vacuum pressure (200 mBar) to remove water [23]. The dried 80% methanolic extract was weighed and percentage of yield was calculated. The dried extract was stored in tight container at −4 °C [22].

The 80% methanol extract was subjected to successive fractionation using solvents of differing polarity (chloroform, ethyl acetate and water). The fractionating solvents were selected based on their differing polarity [24]. Eighty g of the crude extract was suspended in 480 ml of distilled water. Equal volume of chloroform was partitioned in a separatory funnel. The mixture was allowed to form a distinct layer and the chloroform fraction was separated. This was repeated three times. Then the aqueous residue was similarly mixed with equal volume of ethyl acetate and separated. The chloroform and ethyl acetate fractions were concentrated by rotary evaporator and dried by dry oven at 40 °C. The aqueous fraction was lyophilized. The % yields of the dried fractions were calculated and the yields were stored in tight container at −4 °C until used [22].

#### 2.4.1. Ointment formulation

Simple ointment and medicated ointments of 80% methanol extract and chloroform, ethyl acetate and aqueous fractions were prepared as per British Pharmacopeia [25].

To prepare 100 g simple ointment, 5 g of hard paraffin and 5 g of Cetostearyl alcohol were melted in a beaker. A 5 g wool fat and 85 g white soft paraffin were melted in another beaker. The mixtures were then combined and stirred until cooled. A 5% w/w and 10% w/w medicated ointments of the crude extract were prepared by incorporating 5 g and 10 g of the 80% methanol extract respectively with 95 g and 90 g simple ointment bases. Similarly the 5% w/w and 10% w/w ointments of each fraction were prepared by incorporating 2.5 g and 5 g of the aqueous, ethyl acetate and chloroform fraction each into a 47.5 g and 45 g of simple ointment base to get a 50 g medicated ointment of each fraction.

### 2.5. Ethical consideration

The study protocol was approved by the Institutional Ethical Review Board of University of Gondar with a reference number SOP4/51/10. Animals were handled according to international laboratory animal use and care guidelines throughout the experiment.

### 2.6. Acute dermal toxicity study

Acute dermal toxicity was carried out as per the [26] [27].). Three female rats showing normal skin texture were housed individually in a cage and acclimatized to the laboratory condition for seven days prior to the test. Then, the rats were anesthetized by ketamine 80 mg/kg intraperitoneal injection and around 10% of the body surface area fur was shaved from the dorsal area of the trunk 24 h before the study. A limit test dose of 2000 mg/kg of the 10% w/w of the crude extract formulation was applied uniformly over the shaved area for 24 h. During the exposure period, rats were caged individually. At the end of the exposure period, residual test substance was washed out by distilled water and the rats were observed daily for development of any adverse skin reactions for 14 days. The reactions, defined as erythema and edema, were evaluated and graded according to the OECD 404 grades (2002).

### 2.7. Phytochemical screening

The crude extract as well as chloroform, ethyl acetate and aqueous fractions were screened for the presence of alkaloids, saponins, flavonoids, terpenoids, phenols, steroids, glycosides and tannins according to standard test procedures [28–31].

### 2.8. Experimental animals handling, grouping and dosing

Swiss albino mice of both sex (30 ± 5 g and 6–8 weeks of age) and female Wistar rats weighing 180–200 g with 3–4 months of age were obtained from the animal house of University of Gondar and Ethiopian Public Health Institute. The animals were housed in cages under standard conditions with 12 h light and dark cycles. They were provided with standard pellet diet and water ad libitum. They were acclimatized to the laboratory condition for a week before the starting of the experiments. Animals were handled according to international laboratory animal use and care guidelines throughout the experiment. At the end of the experiment, the animals were sacrificed on high dose of halothane inhalation [32].

To evaluate the activities of the crude extract, mice were grouped into four groups for excision, five groups for incision and another five groups for anti-inflammatory activity evaluation each group consisting six mice. For excision wound model, the first group was treated with simple ointment. The second and third groups were treated with 5% and 10% ointments of 80% methanol extract of leaves of *Calpurnia aurea* (Ait.) Benth respectively. The fourth group was treated with nitrofurazone 0.2% ointment. For incision wound model, the first group was left untreated and the second group was treated with simple ointment. Groups III-IV were treated with 5% w/w and 10% w/w ointments of 80% methanol extract respectively. Group V was treated with nitrofurazone 0.2% ointment. For the evaluation of the anti-inflammatory activity of the crude extract, the mice were fasted of food overnight with free access to water. Group I was treated with 2% Tween 80 (1 ml/100 g) orally by oral gavage and served as negative control. Groups from II—IV were treated with 100, 200 and 400 mg/kg p.o of 80% methanol leaf extracts respectively. Mice in group V were treated with indomethacin 10 mg/kg p.o. The extracts and the positive control were suspended in 2% Tween 80. The doses of the crude extract were determined based on previous acute oral toxicity studies on 80% methanol extract of leaves of *Calpurnia aurea* (Ait.) Benth that indicated the LD50 was above 2000 mg/kg [33,34].

For the evaluation of wound healing activity of the chloroform, ethyl acetate and aqueous fractions, similarly 8 groups each containing 6 mice were formed. The first group was treated with simple ointment. Groups II-VII were treated with 5% w/w aqueous, 10% w/w aqueous, 5% w/w ethyl acetate, 10% w/w ethyl acetate, 5% w/w chloroform and 10% w/w chloroform fraction ointments respectively. Group VIII was treated with nitrofurazone 0.2% ointment.

### 2.9. Evaluation of wound healing

#### 2.9.1. Excision wound model

The mice were anesthetized with intraperitoneal 50 mg/kg ketamine and 5 mg/kg diazepam [35]. Then their fur from dorso-thoracic area was removed. A 314 $mm^2$ circular mark was prepared using permanent marker. Then, full thickness of this circular mark was excised using forceps and scissors to form wound. This was considered as day 0. Starting from day one, the mice were treated as described in the grouping and dosing. For the evaluation of wound healing activity of the chloroform, ethyl acetate and aqueous fractions, the same wound area was created and ointments were applied as described in the grouping and dosing. All the preparations were applied daily to the wound area until the wound in the test groups completely healed. The mice were observed for wound closure and measurement was taken every two post wounding days using transparent sheet and permanent marker. The wound healing activities of crude extract and solvent fractions were assessed by the period of epithelialization and percentage of wound contraction [8,36].

The percentage of wound contraction was calculated as follows [8].

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

Where n = number of days i.e. $2^{nd}$, $4^{th}$, $6^{th}$, etc. until the day the wound in the test drug treated groups healed.

The number of days required for the escher to fall off from the wound surface exclusive of leaving a raw wound behind was taken as end point of complete epithelialization and the days required for this was considered as period of epithelialization [37].

#### 2.9.2. Incision wound model

Thirty mice were anaesthetized and their fur was shaved similarly to that in the excision wound model. Three cm long, linear- paravertebral incision was made through the full thickness of the skin on either side of the vertebral column at a distance of 1 cm from the midline. The parted skin was kept together and stitched using black braided silk (no. 00) and a curved needle (no. 11) at the intervals of 1 cm [35]. This wounding day was considered day 0. Starting from day one, the ointments were applied as indicated in the grouping and dosing. The treatments were applied topically once per day for 9 days. The sutures were removed on the $8^{th}$ day of post wounding [36] and the tensile strength was measured on the $10^{th}$ day to measure the extent of healing [38]. It was measured through continuous water flow technique (Fig. 2) by considering the gram of water required to break the skin [39].

The breaking strength of the groups treated with extracts was compared with the standard, simple ointment and untreated control. The percentage of tensile strength was calculated as follows [40].

$$Percent\ tensile\ strength\,(TS)\,of\ treated\ groups = \frac{TS\ treated - TSso}{TS\ treated} \times 100$$

$$Percent\ tensile\ strength\ of\ simple\ ointment = \frac{TSso - TSlu}{TSso} \times 100$$

Where so = simple ointment and lu = left untreated

#### 2.9.3. Anti-inflammatory activity of the crude extract

The acute anti-inflammatory activity of the crude extract against the carrageenan-induced hind paw edema model in mice was determined

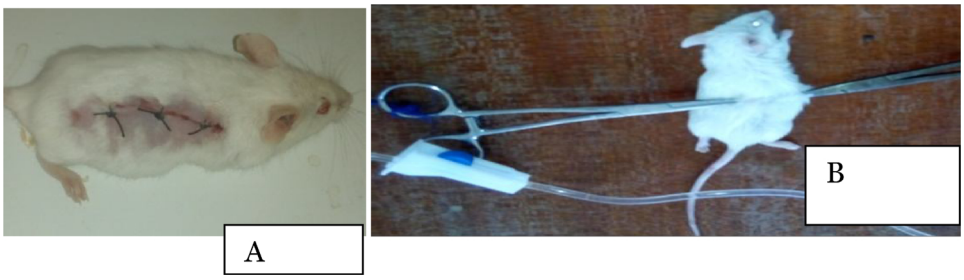


**Fig. 2.** Incision wound creation (A) and tensile strength measurement (B) during the experiment.

according to the standard methods with some modification [41]. The basal volume of the left hind paw of each mouse was determined using Plethysmometer before administration of the substances. Then the treatments were given as indicated in the grouping and dosing. The inflammation in the hind paw was induced by injecting 0.05 ml of freshly prepared carrageenan suspension (1%) in normal saline into the sub plantar-surface of the left hind paw. The change in volume of the injected paw was measured after 1, 2, 3 and 4 h of the administration of carrageenan with the help of Plethysmometer [40]. An increase in paw volume at 1, 2, 3 and 4 h after carrageenan injection was considered as the parameter for measurement of inflammation. The average foot swelling in extract treated mice as well as standard were compared with that of the negative control and the percent inhibition (anti-inflammatory activity) of edema was determined using the following formula [42].

$$Percent\ inhibition\ of\ edema = \frac{PEC - PET}{PEC} \times 100$$

PEC = Paw edema of negative control and PET = paw edema of test groups including the standard.

### 2.10. Statistical analysis

The data obtained from the experiments were expressed as mean ± SEM. The results were statistically analyzed using one-way ANOVA followed by Post Hoc Tukey tests with SPSS version 23 software and considered significantly different at $p < 0.05$ (Table 1).

## 3. Results

### 3.1. Phytochemical screening

Eighty percent methanol extraction of the leaves resulted a 23% of yield of the crude extract and the fractionation produced 10%, 27.5% and 62.5% yield of chloroform, ethyl acetate and aqueous fractions respectively.

The Phytochemical screening test revealed the presence of different secondary metabolites in 80% methanol extract, and aqueous, ethyl acetate and chloroform fractions (Table 2).

### 3.2. Acute dermal toxicity

Topical application of 2000 mg/kg of the 10% ointment formulation of the crude extract produced no edema and erythema on rats. No signs of toxicity and no mortality were observed during the 14 days of observation.

**Table 1**
Formula used for preparation of simple and medicated ointments [25].

| Ingredient | Master formula | Working formula |
|---|---|---|
| Wool fat | 50 | 5 g |
| Hard paraffin | 50 | 5 g |
| Cetostearyl alcohol | 50 g | 5 g |
| White soft paraffin | 850 g | 85 g |
| Total | 1000g | 100 g |

**Table 2**
Results of phytochemical screening of crude extract and solvent fractions.

| Secondary metabolites | 80% methanol extract | Aqueous fraction | Ethyl acetate fraction | Chloroform fraction |
|---|---|---|---|---|
| Alkaloids | + | + | – | – |
| Tannins | + | + | + | – |
| Terpinoids | + | + | + | – |
| Flavonoids | + | + | + | – |
| Saponins | + | + | + | – |
| Phenols | + | + | + | + |
| Steroids | + | – | – | + |
| Glycosides | + | + | + | – |

Where (+) present and (-) absent.

### 3.3. Evaluation of wound healing

#### 3.3.1. Excision wound model

Both 5% w/w and 10% w/w ointments of the crude extract produced increased percentage of wound contraction (Table 3, Fig. 3) and shortened period of epithelialization (Fig. 5) in mice. Groups treated with 10% w/w crude extract ointment and nitrofurazone 0.2% ointment showed significant ($p < 0.05$) wound contraction in day two of post wounding compared to the negative control. Within days 4–8, groups treated with 5% w/w and 10% w/w crude extract ointments and the positive control produced significant ($P < 0.001$) wound contraction compared to the negative control. Starting day 4, the 10% w/w crude extract ointment revealed greater percentage of wound contraction than the standard. However, there was no significant difference in wound contraction between groups treated with 5% w/w, 10% w/w extract and the positive control ointments. Total wound closure in the 10% w/w crude extract ointment and nitrofurazone 0.2% ointment treated groups was observed on day18 post wounding while that of simple ointment treated groups was beyond day 18.

The wound healing activities of the 5% w/w and 10% w/w ointments were further shown by the shorter period of epithelialization. Mice treated with the 5% w/w crude extract ointment showed a significant ($p < 0.05$) reduction in the period of epithelialization compared to the negative control. The 10% w/w crude extract ointment and the standard showed significant ($p < 0.01$) reduction in the period of epithelialization compared to the negative control. There was no apparent difference in epithelialization period between groups treated with 5% w/w, 10% w/w crude extract, and the standard ointments (Fig. 4).

In the excision wound model of the fractions, the mice treated with the aqueous and ethyl acetate fractions ointments revealed wound healing. The percentage of wound contraction of the mice treated with the 10% w/w ointment of the aqueous fraction was significant ($p < 0.001$) in most post wounding days except on day 16 ($p < 0.01$)

**Table 3**
Effect of crude extract on excision wound in mice.

| Days of wound area Measurement | Wound area ($mm^2$) ± SEM (% contraction) | | | |
|---|---|---|---|---|
| | Simple ointment | CALE 5% w/w ointment | CALE 10% w/w ointment | Nitrofurazone 0.2% ointment |
| Day2 | 291.50 ± 12.25 (7.17) | 267.33 ± 5.48 (14.86) | 252.83 ± 12.78$^{a*}$ (19.48) | 251.50 ± 4.47$^{a*}$ (19.90) |
| Day4 | 268.00 ± 10.44 (14.65) | 217.83 ± 4.41$^{a***}$ (30.63) | 205.33 ± 4.56$^{a***}$ (34.61) | 216.50 ± 2.14$^{a***}$ (31.05) |
| day6 | 248.00 ± 9.98 (21.02) | 183.17 ± 6.26$^{a***}$ (41.67) | 164.50 ± 9.11$^{a***}$ (47.61) | 166.00 ± 3.92$^{a***}$ (47.13) |
| Day8 | 207.33 ± 11.96 (33.97) | 151.83 ± 3.36$^{a***}$ (51.65) | 140.50 ± 7.29$^{a***}$ (55.25) | 146.33 ± 6.70$^{a***}$ (53.40) |
| Day10 | 178.00 ± 17.49 (43.31) | 130.42 ± 6.34$^{a*}$ (58.46) | 104.67 ± 8.15$^{a**}$ (66.67) | 117.00 ± 9.38$^{a**}$ (62.74) |
| Day12 | 133.00 ± 16.50 (57.64) | 82.06 ± 5.89$^{a*}$ (73.87) | 63.50 ± 8.50$^{a**}$ (79.78) | 70.83 ± 6.42$^{a**}$ (77.44) |
| Day14 | 83.83 ± 19.20 (73.30) | 38.33 ± 5.72$^{a*}$ (87.79) | 28.33 ± 3.55$^{a**}$ (90.98) | 37.50 ± 7.89$^{a*}$ (88.06) |
| Day16 | 56.00 ± 17.20 (82.17) | 15.91 ± 4.16$^{a*}$ (94.93) | 6.67 ± 4.01$^{a**}$ (97.88) | 7.33 ± 3.45$^{a**}$ (97.66) |
| Day18 | 30.50 ± 11.63 (90.29) | 3.59 ± 1.68$^{a*}$ (98.86) | 0.00 ± .00$^{a**}$ (100.00) | 0.00 ± .00$^{a**}$ (100.00) |

Values are expressed as mean ± SEM (n = 6 mice in each group) and analyzed by one way ANOVA followed by Post Hoc Tukey test; [a] compared to the negative control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Initial wound area was $314mm^2$.

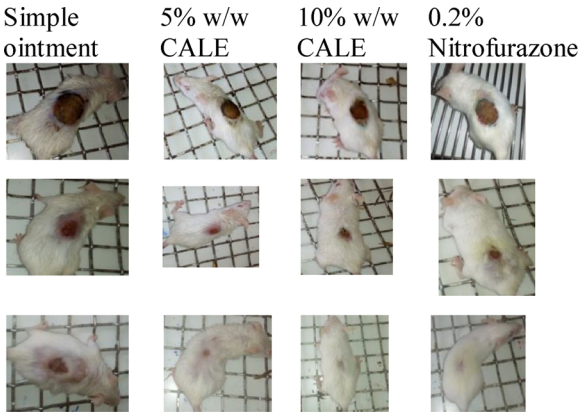


**Fig. 3.** Excision wound on different days.
Where CALE is 80% methanol extracts of leaves of *Calpurnia aurea* (Ait.) Benth.

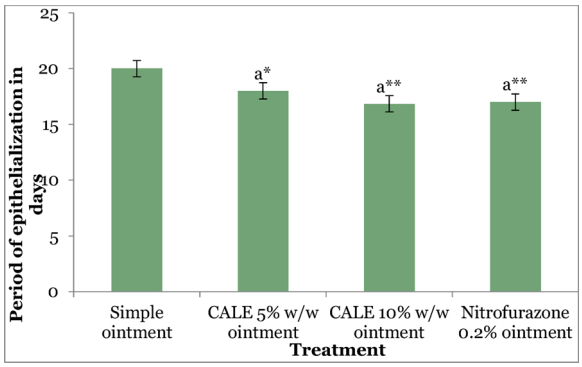


**Fig. 4.** Effect of the crude extract on period of epithelialization.
Values are expressed as mean ± SEM (n = 6 mice in each group) and analyzed by one way ANOVA followed by Post Hoc Tukey test; [a] compared to the negative control; $^{*}p < 0.05$, $^{**}p < 0.01$.

compared to the negative control (Table 4, Fig. 5). The wound contraction of the group treated with 10% w/w aqueous fraction ointment was greater than that of the positive control in all post wounding days except at day six. On the other hand, the mice treated with the 5% w/w and 10% w/w ointments of chloroform fraction showed statistically insignificant wound healing activity compared to the negative control. There was no significant difference in the wound contractions between the mice treated with the three fraction ointments.

AQf = Aqueous fraction, EAF = Ethyl acetate fraction and CHF = Chloroform fraction

Groups treated with the 5% w/w and 10% w/w ointments of each fraction had shorter period of epithelialization when compared to the negative control. However, the period of epithelialization produced by the chloroform fraction ointments could not be statistically significant when compared to the negative control. The periods of epithelialization of mice treated with the 5% w/w and 10% w/w aqueous fraction and the standard ointments were significant ($p < 0.01$) compared to the negative control and that of the two doses of the ethyl acetate fraction were significant ($p < 0.05$) compared to the negative control. There was no significant difference between the treatment doses of the three fractions as well as with the positive control. Mice treated with 10% w/w aqueous fraction ointment had the shortest period of epithelialization (16.33 days) (Fig. 6).

Values are expressed as mean ± SEM (n = 6 mice in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; [a] compared to the negative control; $^{*}p < 0.05$, $^{**}p < 0.01$; AQF = Aqueous fraction, EAF = Ethyl acetate fraction and CHF = Chloroform fraction

### 3.3.2. Incision wound model

In incision wound model, groups treated with 10% w/w and 5% w/w ointments of the crude extract showed significant ($p < 0.001$) increase in breaking strength compared to the control groups (simple ointment and the untreated controls). The increase in tensile strength of simple ointment treated group was not statistically significant when compared to the untreated control. There was no statistically significant difference between the 5% w/w, 10% w/w crude extract ointments and the positive control. The mice treated with 10% w/w crude extract ointment had highest percentage of tensile strength (Table 5).

### 3.4. Anti-inflammatory activity of the crude extract

The 200 mg/kg and 400 mg/kg doses of the crude extract, and the standard drug showed significant ($p < 0.01$) reduction of paw edema after 2 h of carrageenan injection compared to the negative control. After 3 h of carrageenan injection, all doses of the crude extract and the

**Table 4**
Effect of solvent fractions on percentage wound contraction.

| Ointments | Wound area($mm^2$) ± SEM (% contraction) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day2 | Day4 | Day6 | Day8 | Day10 | Day12 | Day14 | Day16 |
| Simple ointment | 294.33 ± 3.17 (6.26) | 269.33 ± 3.21 (14.23) | 237.00 ± 1.90 (24.52) | 195.33 ± 2.23 (37.79) | 146.33 ± 2.62 (53.40) | 109.00 ± 2.49 (65.29) | 74.50 ± 4.28 (76.27) | 33.33 ± 6.09 (84.08) |
| AQF 5% w/w | 276.67 ± 1.54** (11.89) | 242.17 ± 1.25** (22.88) | 198.50 ± 7.32** (36.78) | 144.83 ± 6.96** (53.87) | 110.00 ± 6.91** (64.97) | 70.17 ± 4.64** (77.65) | 30.50 ± 3.26*** (90.29) | 3.67 ± 2.33** (98.83) |
| AQF 10% w/w | 271.17 ± 2.21*** (13.64) | 236.67 ± 2.87*** (24.63) | 192.33 ± 11.82*** (38.75) | 137.00 ± 7.80*** (56.37) | 98.67 ± 4.09*** (68.58) | 64.67 ± 3.83*** (79.41) | 29.67 ± 3.74*** (90.55) | 2.17 ± 2.17** (99.31) |
| EAF 5% w/w | 283.50 ± 3.59 (9.71) | 248.83 ± 3.20* (20.75) | 203.33 ± 4.41* (35.24) | 154.00 ± 11.12* (50.96) | 112.83 ± 8.64** (64.07) | 71.83 ± 10.15** (77.12) | 38.50 ± 8.96** (87.74) | 8.00 ± 3.77* (97.45) |
| EAF 10% w/w | 280.17 ± 6.54 (10.77) | 248.33 ± 9.43* (20.91) | 198.33 ± 7.73** (36.84) | 149.83 ± 12.39** (52.28) | 108.17 ± 6.11** (65.55) | 71.33 ± 8.50** (77.28) | 34.33 ± 5.22*** (89.07) | 4.17 ± 2.71** (98.67) |
| CHF 5% w/w | 285.33 ± 1.26 (9.13) | 254.00 ± 2.70 (19.11) | 210.67 ± 5.68 (32.91) | 164.83 ± 4.36 (47.51) | 123.5 ± 3.47 (60.67) | 90.50 ± 4.26 (71.18) | 54.33 ± 4.46 (82.70) | 22.00 ± 8.49 (92.99) |
| CHF 10% w/w | 285.00 ± 1.84 (9.24) | 251.67 ± 2.17 (19.85) | 216.17 ± 4.42 (31.16) | 164.33 ± 7.15 (47.66) | 123.00 ± 5.94 (60.83) | 89.00 ± 6.01 (71.66) | 51.67 ± 7.80 (83.55) | 17.50 ± 8.54 (94.43) |
| Nitrofurazone 0.2% | 274.00 ± 2.32* (12.74) | 245.33 ± 1.56** (21.87) | 192.17 ± 1.30*** (38.80) | 145.50 ± 2.09** (53.66) | 103.33 ± 5.36*** (67.09) | 68.83 ± 7.30** (78.08) | 37.83 ± 4.31** (87.95) | 5.50 ± 3.50* (98.25) |

Values are expressed as mean ± SEM (n = 6 mice in each group) and analyzed by one way ANOVA followed by Post Hoc Tukey test; * compared to the negative control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; AQF = Aqueous fraction, EAF = Ethyl acetate fraction, CHF = Chloroform fraction. Initial wound area was $314mm^2$.

standard drug showed significant edema reduction ($p < 0.01$) compared to the negative control. Maximum anti-inflammatory effect (% inhibition of edema) of the crude extract was observed after 4 h post injection by the 100, 200 and 400 mg/kg oral doses, with respective values of 55.16%, 56.68% and 65.5%. The standard drug showed maximum percent of edema inhibition four hours after carrageenan injection with 86.36% of edema inhibition (Table 6).

## 4. Discussion

Medicinal plants have great potentials and have been shown to be beneficial in wound care. Medicinal plants promote the rate of wound healing with minimal pain, discomfort, and scarring to the patient [43]. Ointment formulations of medicinal plants could achieve wound healing [44].

The ointments prepared from the crude extract showed rapid wound contraction. This enhanced wound contraction by the crude extract ointments might related to the ability of plant extracts to promote proliferation of epithelial cells [45]. In addition, the wound contraction effect of ointments of the crude extract might be associated with their mitogenic activity which enhances fibroblast motility and its cellular proliferation as well as sequential transformation to myofibroblast that facilitate wound contraction during wound healing [35].

Previous studies indicated that 80% methanol extract of the leaves of *Calpurnia aurea* (Ait.) Benth had antibacterial activities against common wound infecting bacteria such as *Staphylococcus aurous*, *Escherichia coli* and *Pseudomonas aeruginosa* [21] and this antimicrobial activity might partly contribute to the wound healing effect of the plant by eliminating infection and allowing initiation of natural tissue repair process.

The reduced the period of epithelialization produced by the ointments could be due to rapid wound contraction as contraction shortens the distance for migrating keratinocytes [46]. Additionally, the shorter period of epithelialization in the groups treated with the crude extract ointments might be related with the antibacterial activity of the leaves extracts of the experimental plant described earlier. The role of the antibacterial activity of the crude extract in reducing the period of epithelialization might be related with bacterial endotoxin load reduction in the wound area. Endotoxins reduce proliferative capacity of fibroblasts and epithelial cells [47]. The ointments might reduce bacteria and endotoxins allowing fibroblasts and epithelial cells to proliferate. Reduction in the epithelialization period by the extract may be attributed to its ability to enhance contractile property of myofibroblasts and proliferate epithelial cells around the wound [48].

Moreover, the increased wound contraction and the reduced period of epithelialization in mice treated with the crude extract ointments could be attributed with the presence of terpenoids. Terpenoids are known to promote wound healing process, mainly due to their astringent and antimicrobial activities which seem to be responsible for wound contraction and reduced period of epithelialization [49].

Ointments prepared from solvent fractions had differing wound healing activities in excision wound model. Fast contraction rate and short period of epithelialization in mice treated with the aqueous fraction ointments might be related with the accumulation of secondary metabolites such as alkaloids, tannins, terpinoids, flavonoids, saponins, phenols. Flavonoids could promote in wound healing through their astringent and antibacterial activities [50]. Alkaloids might facilitate wound healing through preventing inflammation by blocking the metabolic pathway of arachidonic acid [51]. The findings of this study on wound healing activity of the aqueous and chloroform fractions of 80% methanol extract of the leaves of the experimental plant were comparable with the findings of other studies [52]. The ethyl acetate fraction also promoted wound healing with comparable findings with the results of other studies [53]. The observed insignificant wound healing activity of the chloroform fraction might be due to absence of many secondary metabolites or lower concentration of the secondary metabolites to achieve wound healing activity.

The tensile strength indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue [54]. The increased tensile strength may be due to collagen synthesis, maturation, angiogenesis and stabilization of fibers [55]. Thus, the crude extract might have roles in collagen synthesis, maturation and stabilization.

Abundant ROS damage extracellular structure proteins, lipids, and DNA and stimulate signal transduction pathways to prolong the inflammatory phase of wound healing [56]. Therefore, elimination of ROS could be an important strategy in healing of wounds. Previous studies indicated that methanol extract of the leaves of *Calpurnia aurea* (Ait.) Benth possess antioxidant properties [9]. This antioxidant effect might enable the leaves of the experimental plant inhibit lipid peroxidation, prevent cell damage and increase collagen fibrillary endurance of the healing wound so that facilitating the wound healing process. This could produce rapid wound contraction and shorter period of epithelialization.

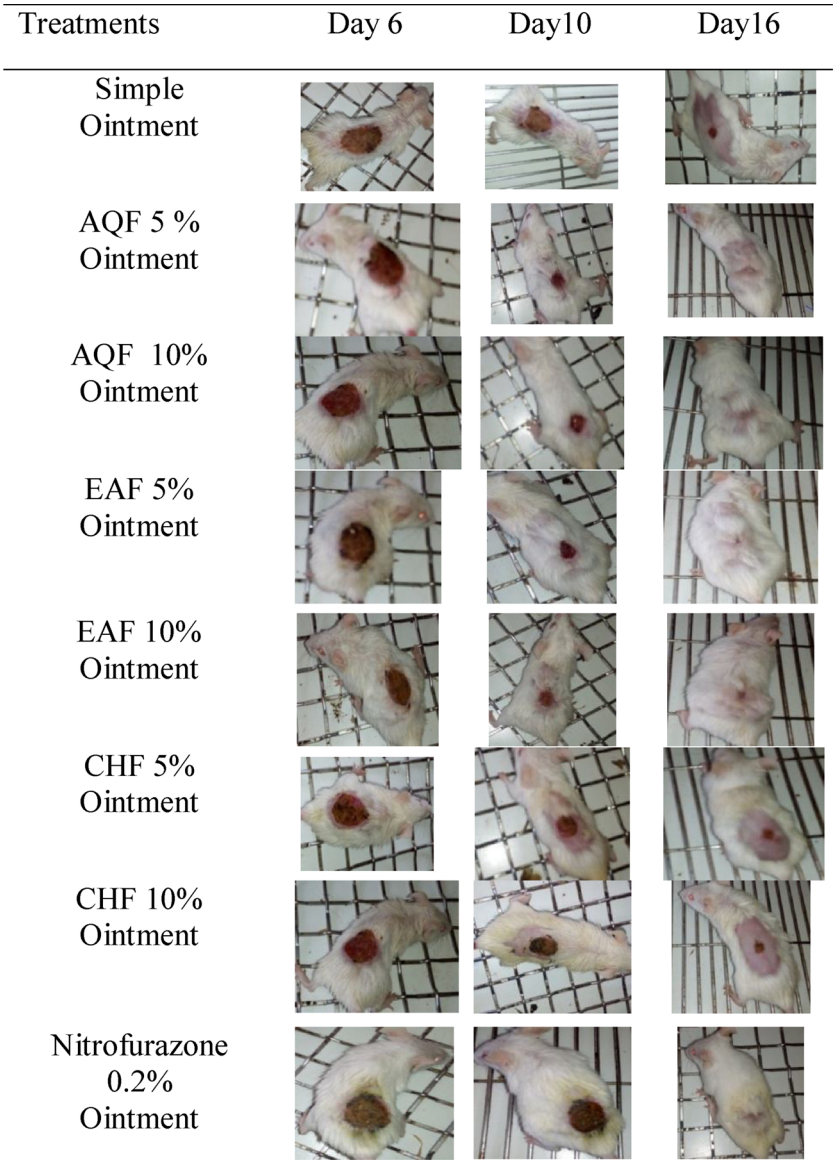


**Fig. 5.** Excision wound on mice treated with solvent fractions ointments.

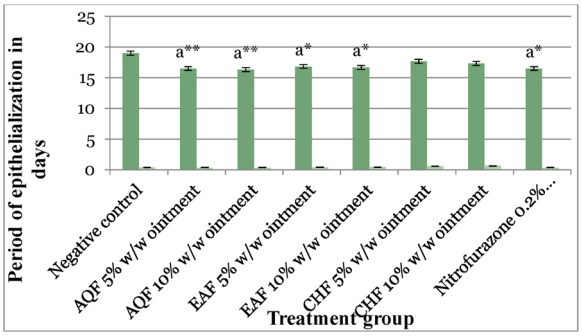


**Fig. 6.** Effect of solvent fractions on period of epithelialization.

Since long period in inflammatory phase results in retardation of healing, anti-inflammatory activity is essential for the wound healing process [57]. The experimental plant revealed anti- inflammatory activity in the carrageenan induced paw edema model and this effect of the extract might facilitate the wound healing activity. Previous studies reported the wound healing and anti-inflammatory activities of medicinal plants including *Gmelina arborea* and *Hemigraphis colorata* [58] and *Rumex abyssinicus* [40]. Prolonged inflammation leads to an increased level of neutrophil derived matrix metalloproteinases that can degrade the ECM [59]. Additionally, prostaglandins are involved in chemotaxis of leukocytes mainly neutrophils that contribute to inflammatory response by producing oxygen derived free radicals that damage wound tissue [60]. Thus, prostaglandin inhibition benefit

**Table 5**
Effect of the 80% methanol extract ointments on tensile strength.

| Group | Tensile strength in gram | Percent tensile strength |
|---|---|---|
| Untreated | 166.83 ± 9.361 | – |
| Simple ointment | 188.83 ± 9.645 | 13.19 |
| CALE 5% w/w ointment | 271.67 ± 8.962$^{a*b*}$ | 43.87 |
| CALE 10% w/w ointment | 289.17 ± 6.019$^{a*b*}$ | 53.13 |
| Nitrofurazone 0.2% ointment | 287.33 ± 15.645$^{a*b*}$ | 52.16 |

Values are expressed as mean ± SEM (n = 6 mice in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; [a] compared to the untreated group, [b] compared to the simple ointment treated group; [*] $p < 0.001$; CALE = Eighty percent methanol extract of leaves of *Calpurnia aurea* (Ait.) Benth.

**Table 6**
Anti-inflammatory effect of the crude extract on carrageenan-induced paw edema.

| Treatment | Basal volume | Increase in paw volume (ml) after carrageenan injection (mean ± SEM)/Percent inhibition of edema | | | |
|---|---|---|---|---|---|
| | | 1 Hour | 2 Hour | 3 Hour | 4 Hour |
| 2% Tween 80 | 0.108 ± .007 | 0.178 ± .007 | 0.182 ± .008 | 0.172 ± .008 | 0.157 ± .008 |
| CALE 100 mg/kg | 0.108 ± .007 | 0.172 ± .010 (9.43) | 0.160 ± .006 (29.56) | 0.140 ± .005* (52.47) | 0.127 ± .003* (55.16) |
| CALE 200 mg/kg | 0.103 ± .008 | 0.160 ± .006 (19.00) | 0.143 ± .004* (45.50) | 0.133 ± .003* (55.02) | 0.123 ± .004* (56.68) |
| CALE 400 mg/kg | 0.103 ± .008 | 0.153 ± .007 (28.57) | 0.140 ± .005* (50.00) | 0.130 ± .007* (59.97) | 0.120 ± .005** (65.50) |
| Indomethacin 10 mg/kg | 0.117 ± .004 | 0.153 ± .001 (47.71) | 0.145 ± .008* (61.44) | 0.137 ± .007* (70.02) | 0.123 ± .004* (86.36) |

Values are expressed as mean ± SEM (n = 6 mice in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; * $p < 0.01$, ** $p < 0.001$; BV = Basal paw volume; CALE = Eighty percent methanol extract of leaves of *Calpurnia aurea* (Ait.) Benth.

wound healing by reducing damaging effect of neutrophils. Hence this anti-inflammatory activity of the experimental plant could promote the wound healing process.

Secondary metabolites could facilitate wound healing either individually or through their additive effects [61]. Tannins enhance wound healing by improving regeneration and organization of the new tissue through their astringent and antioxidant properties [61]. Flavonoids reduce lipid peroxidation by preventing or slowing the onset of cell necrosis and by improving vascularity and have astringent and antimicrobial properties [39]. Phenolic compounds have antioxidant, anti-inflammatory, and antimicrobial activities [62].

Hence, the observed wound healing effect of the crude extract as well as the aqueous and ethyl acetate fractions might be related with the presence of respective secondary metabolites. Although the chloroform fraction was screened for the presence of phenols and steroids, it could not produce significant wound healing effects. This might be related to either lower concentrations of the secondary metabolites that could be detected present in the qualitative phytochemical screening tests or loss of additive effect with other secondary metabolites.

Carrageenan-induced paw edema is a well established animal model to assess the anti-inflammatory effect of natural products. Edema formation due to carrageenan in paw occurs biphasically. The initial phase (1 h–1.5 h) is predominately a non-phagocytic edema followed by a second phase (2–5) h with increased edema formation that remains up to 5 h. During the initial phase, mediators such as histamine, serotonin and bradykinin induce edema through increasing vascular permeability. Overproduction of cyclooxygenase leading to increased synthesis of prostaglandins and production of oxygen free radicals accompanied with massive infiltration of neutrophils induce edema in the late phase [63,64].

The 200 mg/kg and 400 mg/kg doses of the 80% methanol extract of the leaves of *Calpurnia aurea* (Ait.) Benth inhibited inflammation after 2 hours of carrageenan injection. This might indicate that the leaves of the experimental plant could have similar mechanism of action of drugs that inhibit prostaglandin synthesis. This in vivo anti-inflammatory effect of the 80% methanol extract of the leaves supports the in vitro anti-inflammatory activity of acetone extract of leaves of the experimental plant [65].

The anti-inflammatory effect of the crude extract might be associated with the activities of secondary metabolites. Flavonoids can significantly inhibit a number of inflammatory mediators and prevent the synthesis of prostaglandins [66]. Terpenoids inhibit phospholipase A2 and block the metabolism of arachidonic acid. Alkaloids prevent inflammation through blocking the metabolic pathway of arachidonic acid [51].

## 5. Conclusion

The crude extract exhibited wound healing and anti-inflammatory activities in mice. The better wound healing effect elicited by the aqueous fraction corroborates with the folkloric practice that the healers crush the leaves of *Calpurnia aurea* (Ait.) Benth with water for topical application. The results of this study support the medicinal use of leaves of *Calpurnia aurea* (Ait.) Benth for wound healing.

## Financial disclosure

Amhara Regional State Health Bureau was the financial source to conduct this research.

## Authors' contributions

Getachew Ayal carried out the study, designed and conducted all laboratory experiments; analyzed and interpreted experimental results and finally prepared the manuscript. Dr. Wubayehu Kahaliw and Assefa Belay supervised the study. All authors read and approved the manuscript.

## Ethical consideration

The study protocol was approved by the Institutional Ethical Review Board of University of Gondar with a reference number SOP4/51/10. Animals were handled according to international laboratory animal use and care guidelines throughout the experiment.

## Conflict of interest

The authors of this research article declared that there is no conflict of interest.

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