

## IN VIVO EVALUATION OF WOUND HEALING AND ANTI-INFLAMMATORY ACTIVITY OF METHANOLIC EXTRACT OF ROOTS OF *CENTAUREA AFRICANA* (L.) IN TOPICAL FORMULATION

MOHAMED ZAOUANI<sup>1\*</sup>, AREZKI BITAM<sup>2</sup>, AHCEN BAZ<sup>3</sup>, YASMINE BENALI<sup>4</sup>, MERIEM HIND BEN-MAHDI<sup>1</sup>

<sup>1</sup>Research Laboratory of Health and Animal Production, National Higher Veterinary School of Algiers, Issad Abbes, Oued Smar, Algiers, Algeria. <sup>2</sup>Department of Food Technology and Human Nutrition, Agronomic Higher National School El-Harrach, Algiers, Algeria.

<sup>3</sup>Department of Biology, Laboratory of Animal Physiology and Cell Signaling, ENS Kouba, Algiers 16000, Algeria. <sup>4</sup>Department of Veterinary Pathology, Laboratory of Cytopathology Veterinary, Pasteur Institute of Algiers, Algeria. Email: m.zaouani@ensv.dz

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### ABSTRACT

**Objective:** The present study was to evaluate the anti-inflammatory and wound healing activities of methanolic extract of *Centaurea africana* roots in albino Wistar rats.

**Methods:** Following extraction of the *C. africana* roots with 80% methanol, the extract was formulated as an ointment (5% and 10% w/w). The ointment was then evaluated for wound healing activity using excision and incision wound models. Parameters, including wound contraction, epithelization time, histopathologically, and hydroxyproline content, were determined using the excision model, whereas tensile strength was measured from the incision model. In parallel, edema of the ear, locally induced by croton oil, was studied for the assessment of anti-inflammatory activity.

**Results:** Wound treated with 5% and 10% methanolic extract ointment exhibited a significant wound healing activity in both models as evidenced by increased wound contraction, shorter epithelization time, higher tissue breaking strength, and increased hydroxyproline content. The methanolic extract also produced dose-related significant reduction ( $p < 0.001$ ) of inflammation.

**Conclusion:** Results of the present study revealed that *C. africana* displays remarkable wound healing and anti-inflammatory activities.

**Keywords:** Anti-inflammatory activity, *Centaurea africana* roots, Methanolic extract, Incision and excision wound model.

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### INTRODUCTION

The plant world is an inexhaustible source of biologically active molecules to discover, and the pharmaceutical chemistry builds on these models in its designs requiring scientific validation. Several drugs obtained from plant sources are known to increase the healing of different types of wounds [1]. Moreover, herbal medicine is less toxic and less costly when compared to synthetic drugs [2]. Among these plants, we cite a plant which is traditionally used for the treatment of wounds, cuts, and burns in Algeria, *Centaurea africana* of the family Asteraceae, endemic of North Africa, hence the name africana [3]. This plant was the subject of many botanical and chemical investigations while its biological activities are still little studied. The species of *Centaurea* revealed their richness in secondary metabolites such as the lactones, the sesquiterpenes [4,5], sterols [6,7], the phenolic compounds of flavonic type and at a lesser degree, and the alkaloids [8]. Similarly, they have demonstrated their activities: Antirheumatic, anti-inflammatory, digestive, antibacterial, and antipyretic [9]. We are unable to find any information on the wound healing properties of this plant. The aim of our work is, therefore, an attempt to assess the healing and anti-inflammatory properties by topical of a methanolic extract-based ointment, of the roots of *C. africana*, at two concentrations: 5% and 10%.

### METHODS

#### Plant material

The plant material is composed of the roots of *C. africana* var. africana (Bonnet) M, harvested in June 2014, in the region of Kabylie (Draa El Mizan), located at 42 km Southwest of TiziOuzou and at 110 km Southeast of Algiers, Algeria. The plant has been identified by Doctor

Belmokhtar, Botanist Researcher at the Laboratory of Botany located at the Faculty of Pharmacy in University of Algiers. A specimen of this plant has been authenticated by comparison with that recorded in the herbarium of the National Superior School of Agronomy of Algiers.

The roots have been dried, sheltered from the sun and at room temperature, then finely grounded, and stored in sterile vials and sealed.

#### Preparation of extract and ointment

A volume of 50 g of powder has been put in maceration in a mixture of 500 ml of methanol-water solution (80:20 v/v) then with stirred for 72 hrs at room temperature and protected from light. After filtration, the obtained extract has been subjected to evaporation under reduced pressure using a Rotavapor of type (Heidolph Laborota 4000) at 40°C then lyophilized by using the lyophilizer (VirTis benchtop K) for 12 hrs. The extraction yield has been calculated from the powder of the lyophilized *C. africana* roots.

Two ointments, of 5% and of 10% concentration, were prepared. The formula and proportions used for the preparation of the ointment are reported in Table 1.

The simple ointment of the 80% methanolic extract has been prepared following the formula indicated in Table 1. It should be noted that this formula is described in the British Pharmacopoeia [10]. It must be signaled that the three ointment preparations weigh 200 g each, with 5% and 10% w/w and the simple vehicle ointment, with 0% w/w, serves as a witness. The extract was prepared using the reduced formula the master formula (MF) (Table 1).

The four key steps of the adapted operative mode in this study are the weighing of the different raw materials, according to the quantities mentioned in the formula indicated in Table 1 followed by melting and mixing. The first mixture consists of melting, with stirring, the liquid paraffin, and the cetyl alcohol at 80°C. Thereafter, we proceeded with the preparation of the second mixture and this on the eve of the formulation by fluidizing the lanolin and the petrolatum by using a hot plate. The two resulting mixtures have been homogenized by a propeller stirrer IKA (Labortechnik Staufen, Germany). The ointment has been obtained by adding the principle active, 10 g and 20 g, of an 80% methanolic extract in 190 g and 180 g, respectively, in the base ointment. The mixture has been cooled prior to 50°C and maintained stirred at 80 rev/m to obtain a homogeneous product. In preparing the vehicle ointment, 200 g of the base ointment was collected and treated in the same way with which the ointment, without active ingredient (MF), has been formulating [11].

#### Preliminary phytochemical screening

Secondary metabolites existing in the extract of the powder of the studied roots have been identified by characterization tests based on the coloration reactions and/or precipitation according to the standard procedure previously described [12,13]. The tannins and polyphenols have been highlighted by the ferric chloride test. The Liebermann-Burchard test allowed the characterization of the triterpenes and steroids. The appearance of foam after shaking the extract allowed us to identify the saponins. It must be signaled that the flavonoids have also been characterized [14].

#### Evaluation of acute toxicity

In this evaluation, two processing modes have been studied; one by contact and the other by ingestion of the *C. africana* powder roots.

#### Experimental animals

The study has been performed on albinos Wistar rats which were purchased from Pasteur Institute of Algiers, aged about 3 months, and weighing (200±20 g). After an adaptation period of 10-day, the witness animals and treated animals have been isolated in cages with water and food. The food used is a standard diet, supplied by the National Office for Animal's Feed, situated at Bejaia in Algeria. The study animals were used to identify different activities such as the acute toxicity, anti-inflammatory activity, and the healing wound activity, which have been subjected to identical experimental conditions of temperature (21°C±2°C), hygrometry (55-60%), and the photoperiod with a 12 hrs daylight/dark cycle. The experiments have to be carried out as per instructions, partly from the Institutional Animal Ethical Committee (I.A.E.C), and another part from the National Instructions on the Care and Use of Laboratory Animals.

#### Acute toxicity study

The acute toxicity test has been performed according to the toxic class method 423 as per the Organization for Economic Cooperation and Development (OECD) guidelines. The methanolic roots extract of *C. africana* was given orally in a single dose of (2000 mg/kg) body weight to a treated group of 10 rats. The witness group (with n=10) received only water. The rats have been placed under observation continuously for 24 hrs after treatment and for 14 days for changes in signs and symptoms and mortality.

#### Acute dermal toxicity

The evaluation of dermal acute toxicity has been carried out according to the toxic class method 402 as per the OECD guidelines. A total of 10 (5 females and 5 males) rats were used. Animals showing normal skin texture were housed individually in a cage and acclimatized to the laboratory condition for 5 days prior to the test. Around 10% of the body surface area fur was shaved 24 hrs before the study from the dorsal area of the trunk of the test animals. A limit test dose (2000 mg/kg) of the 10% formulation was applied uniformly over the shaved area for 24 hrs. The rats were maintained under observation during a period of 14 days to detect any possible skin reaction and to report any registered mortality.

#### Anti-inflammatory activity

Topical anti-inflammatory activity was evaluated as inhibition of the croton oil-induced ear edema in rats [15]. Male Wistar rats were used for the study and divided into four groups of six rats each. Group-I: Control group, applied with sample ointment base+croton oil solution, Group-II: Standard Voltaren® Emulgel 1% (diclofenac sodium topical gel) novartis+croton oil solution. Group-III: Ointment extract 5%+croton oil solution and Group-IV: Ointment extract 10%+croton oil solution. For tests in rats, the following croton oil solution was prepared (v/v): 4 parts croton oil, 10 parts ethanol, 20 parts pyridine, and 66 parts ethyl ether. The standard drug and the test extract were dissolved in this solution. Formulation of each extract was applied after application of croton oil irritant solution. The test compounds and standard drug diclofenac were dissolved in a concentration of 10 mg/ml in the irritant solution. Irritant solution (0.02 ml) was then applied on both sides of the right ear (RE). The left ear remained untreated. The irritant solution was applied under ether anesthesia. 6 hrs later, the animals were killed by cervical dislocation; the right and left ears of each animal were removed. The left ear (LE) was considered as a control. Circular sections were taken with a cork borer (diameter of 7 mm) and weighed. The anti-inflammatory effect has been determined by expressing the change in weight of the treated ear as compared to the untreated ear and also the control group. The data are expressed as mean±standard error of the mean.

The edematous response was measured as the weight difference between the two plugs.

$$\% \text{ of edema} = \frac{\text{RE} - \text{LE}}{\text{LE}} \times 100$$

The percentage of reduction of edema in the treated rats (T) compared with controls (C) has been calculated by using the following relation:

$$\% \text{ reduction of edema} = \frac{\% \text{ edema C} - \% \text{ edema T}}{\% \text{ edema C}} \times 100$$

#### Excision wound model

The healing wound activity from the excision model in rats has been studied by the daily application of the prepared ointment with based methanolic extract at (5%, 10%), the Cycatril® reference product and of the vehicle ointment without extract on the circular wounds of 2.5 cm in diameter (500 mm<sup>2</sup> surface) and of 2 mm of the depth at the dorsal-cervical region on previously shaved and anesthetized rats by injection of Ketamine® by intraperitoneally at a dose of (150 mg/kg) [16].

The animals were divided into four groups in individual cages; each group contains six animals. Group-I (control) received an application of sample ointment base. Group-II (standard) received the application of standard drug (Cycatril®). Group-III received the application of formulation at 5% ointment and Group-IV received formulation at 10% ointment. The various parameters such as wound contraction percentage, epithelialization period, hydroxyproline content, and histopathology of granular tissues were evaluated.

The percentage of wound closure has been calculated using the following formula [17].

$$\text{Wound closure on day N} = \frac{\text{Area on day 0} - \text{Open area on day N}}{\text{Area on day 0}} \times 100$$

#### Incision wound model

The distribution of the different groups has been made the same way than in the case of the skin excision model. After waxing and anesthesia by Ketamine® (150 mg/kg), a longitudinal incision of 5 cm length was made through the skin and cutaneous muscle at a distance of about 1.5 cm from the midline of each side on the back depilated immediately

after, the wound was connected with staples at 0.5 cm of intervals to ensure its proper closure. The ointment with methanolic extract at (5% and 10%), the Cycatril® reference product and the vehicle ointment have been applied once daily for 9 days [18]. When wounds were cured thoroughly, the sutures were removed on the 9<sup>th</sup> day, and tensile strength was measured with a tensiometer using Lee method [19].

#### Hydroxyproline content

The hydroxyproline is a basic constituent of collagen [20]. The collagen content of granulation tissue has been determined by estimating the content of hydroxyproline [21]. On the 12<sup>th</sup> day of observation, the animals from each group were sacrificed through an overdose of anesthesia of ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (25 mg/kg). Once the samples of the scar were removed, the hydroxyproline content of the granulation tissue has been measured after hydrolysis of the samples in the HCl-6 N at 105°C for 18 hrs. The hydroxyproline was oxidized by chloramine-T (1.4% w/v in acetate citrate buffer, pH 6.0) and incubated for 20 minutes in the reactant, Ehrlich type, à 60°C. After acid extraction with toluene, the hydroxyproline in each tissue sample was deduced from a standard calibration curve. The hydroxyproline rate has been expressed in milligrams per gram of dry tissue weight.

#### Statistical analysis

The statistical analysis was performed by using one-way analysis of variance followed by Dunnett's test for individual comparison of groups with control.  $p < 0.001$  was considered as significant.

#### Histopathological studies

On the 12<sup>th</sup> day, the granulation tissues of all groups of animals, in the excision model, were quickly removed, washed in 0.9% NaCl solution, fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections of 5 µm have been colored with hematoxylin and eosin.

The histopathological examination was applied to determine the regeneration of collagen, the infiltration of the fibroblasts, the neovascularization, and the epithelialization in the zone of the wounds compared to the control group and treated group with Cycatril® reference product.

### RESULTS

#### Qualitative phytochemical screening

The results of the preliminary phytochemical screening confirm the presence of several chemical groups that might be responsible for the pharmacological activities. The results revealed the presence of the flavonoids, tannins, anthocyanins, leucoanthocyanin, coumarin, glycosides, terpenoids, quinones, and saponins. The extraction yield was evaluated 9.7%.

#### Acute dermal toxicity study

No mortality signs or even clinical signs of toxicity such as the irritation, sensitivity, redness, and the inflammation were registered during the 14 days of observation. This allowed us to estimate that the acute toxicity found is  $>2000$  mg/kg.

#### Acute oral toxicity study

No mortality was recorded in these animals up to 14 days. Thus, the methanol extract was found to be non-toxic up to a dose of 2000 mg/kg body weight.

#### Anti-inflammatory activity

The results of the anti-inflammatory activity of the methanolic extract of the roots of *C. africana* at a concentration of 5% and 10% are illustrated in Table 2. The reference drug was found to be comparatively more potent as compared to the formulated ointment of 5% and 10%. In addition, the percentage reduction of edema is higher in the case of the ointment at 10% than that of 5%. The anti-inflammatory activity is concentration dependent.

#### Incision wound model

Table 3 shows the effects of 5% and 10% *C. africana* topical ointment on wound healing activity in rats inflicted with incision wound. In our study, the tensile strength of skin was found to be significantly increased ( $p < 0.001$ ), in standard Cycatril®, 5% extract ointment and 10% extract ointment groups when compared with the control group of animals. The tensile strength of animals treated with the standard drug was higher than the 5% extract-treated animals although no apparent difference was detected with 10% formulation of the extract. These observations of incision wound model confirm the prohealing effect of the application of the ointment with based methanolic extract as observed in excision wound model.

#### Excision wound model

The methanolic extract 80% of the roots of *C. africana* formulated by ointment was presented in Table 4 and Fig. 1. It was observed that the wound contracting ability of the extract ointment in both concentrations were significantly greater ( $p < 0.001$ ) on days 4, 8, 12, and 16 compared to the control group (sample ointment base). This was shown by the percentage of wound closure and epithelialization period that indicates the rate at which wound healing was progressed. The animals treated with ointment 10% of methanolic extract shown the healing of wound completed within 17 days compared to standard reference Cycatril® Group-II took 18 days for the complete wound. The epithelialization period was found to be less ointment extract 5% methanolic extract (20 days). All the recorded observations confirmed that the extract ointment at 10% is found to be the most potent.

#### Effect on hydroxyproline content in rats

The hydroxyproline content of the granulation tissue followed the same pattern as that of wound contraction (Table 5). The highest concentration of hydroxyproline is seen in the case of animals, which received with ointment extract 10%, was comparatively more significant. Followed by animals treated with Standard Cycatril® and animals treated with ointment extract 5%. The animals which receiving no treatment have the lowest content of hydroxyproline.

#### Histopathology

The histopathology study of excision wound skin at day 12 stained with hematoxylin and eosin ( $\times 25$ ) granulation tissue sections is presented in Fig. 2. Control rats (Fig. 2a) showed the presence of acute inflammatory, fibroblastic connective tissue, and very less number of blood vessels. The collagen fibers could not be distinguished to indicate incomplete healing of the wound in the control animals. Granuloma tissue of the animals treated with ointment extract 5% (Fig. 2b) showed incomplete epithelial cells, fibrosis, and mild edema with lymphoid cells. Ointment extract 10% (Fig. 2c)-treated animals showed a moderate amount of collagen and fibroblasts and increased number of blood vessels. The reference ointment (Cycatril®) (Fig. 2d) showed a large amount of granulation tissue, re-epithelialization, and a small number of inflammatory cells.

### DISCUSSION

The pharmacological potentialities of methanolic extract of the roots of *C. africana* determined in this study are due principally to the presence of this extract of secondary metabolites such as flavonoids, coumarin, terpenoids, and tannins. These have been identified from the preliminary phytochemical screening of the methanolic extract of the study as indicated above. Indeed, tannins and flavonoids are reported to inhibit prostaglandin synthesis [22] and also known for their role in the strengthening of the healing process of the wound, mainly due to their antimicrobial property [23] and also the flavonoids and triterpenoids are also known as active compounds promoting the healing process because of their astringent and antimicrobial properties [24,25].

In the present study, the topical application of the ointments of the methanolic extract of the roots of *C. africana* promoted the healing of the wound as evidenced by the increase of wound contraction during the

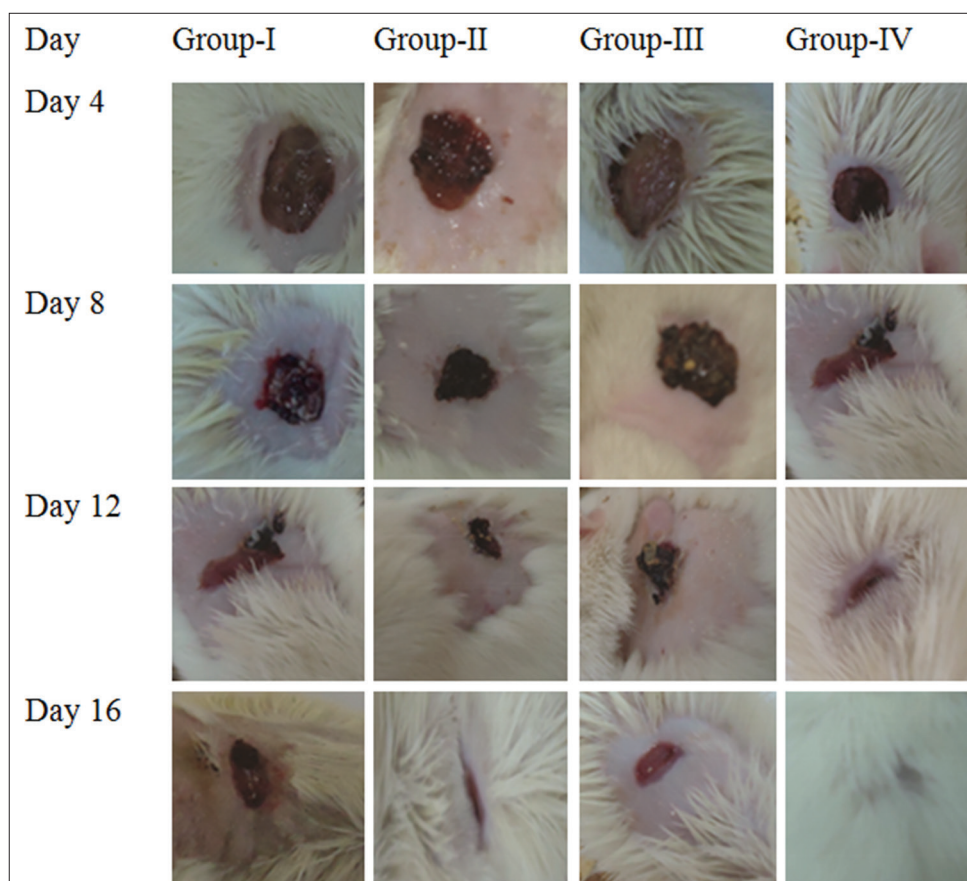


Fig. 1: Photographical representation of wound healing in rats subjected to skin excision wounds on different days: Group-I: Control sample ointment base; Group-II; Standard reference Cycatril®; Group-III: Ointment extract 5%; Group-IV: Ointment extract 10%

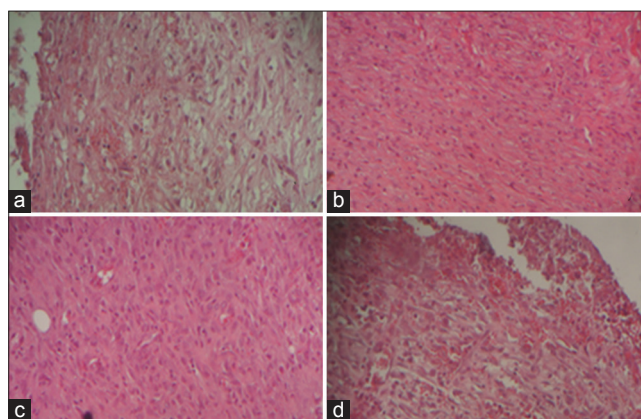


fig. 2: Histopathological evaluation of wound healing process in different treatment groups: (a) Control (ointment base), (b) standard (Cycatril®), (c) ointment (extract 5%), (d) ointment (extract 10%)

Table 1: The formula used for preparing the ointment

Composants	MF (g)	RF (g)
Wool fat	45	9
Hard paraffin	150	30
White soft paraffin	800	160
Cetostearyl alcohol	5	1
Total	1000	200

MF: Master formula, RF: Reduced formula

Table 2: Effect of topical application of the ointment formulated from 80% methanol extract of the roots of *C. africana* on edema induced in the rat by croton oil

Groups	Edema (mg)	Percentage inhibition of edema
Control Group-I	46.81±4.48	-
Standard diclofenac Group-II	12.13±1.31*	73.93
Ointment extract 5% Group-III	18.02±1.00*	61.26
Ointment extract 10% Group-IV	12.71±0.85*	72.68

\*p<0.001 compared to control. Values are mean±SEM (n=6). *C. africana*: *Centaurea africana*, SEM: Standard error of mean

Table 3: Effect of topical application of the ointment formulated from 80% methanol extract of the roots of *C. africana* on tensile strength of incision wound

Groups	Incision tensile strength (g)
Control Group-I	451.67±7.70
Standard Cycatril® Group-II	595.00±18.71*
Ointment extract 5% Group-III	532.00±16.87*
Ointment extract 10% Group-IV	620.00±19.80*

\*p<0.001 compared to control. Values are mean±SEM (n=6). *C. africana*: *Centaurea africana*, SEM: Standard error of mean

proliferative phase. The wound contraction enhances closure by pulling the edges of the wound toward the center; this centripetal movement of wound margin is believed to be due to the activity of myofibroblast [26].

**Table 4: Effect of topical application of the ointment formulated from 80% methanol extract of the roots of *C. africana* on wound contraction and epithelization period of excision wound model**

Groups	Percentage of wound contraction				Day of epithelization
	4 <sup>th</sup> day	8 <sup>th</sup> day	12 <sup>th</sup> day	16 <sup>th</sup> day	
Control Group-I	20.74±8.05	30.56±7.02	43.69±6.12	60.53±8.71	24.00±0.37
Standard Cycatril® Group-II	34.04±3.20*	62.53±3.13*	81.66±14.98*	92.35±2.43*	18.00±0.49*
Ointment extract 5% Group-III	26.32±3.98*	56.51±3.50*	73.37±3.02*	80.11±4.74*	20.67±0.46*
Ointment extract 10% Group-IV	33.89±4.63*	62.22±3.57*	82.20±3.17*	97.99±5.43*	17.00±0.49*

\*p<0.001 compared to control. Values are mean±SEM (n=6). *C. Africana*: *Centaurea Africana*, SEM: Standard error of mean

**Table 5: Hydroxyproline content of excision wounds following the topical application of the ointment formulated from 80% methanol extract of roots of the *C. africana***

Groups	Hydroxyproline (mg/g tissue)
Control Group-I	108.91±1.77
Standard Cycatril® Group-II	169.68±3.34*
Ointment extract 5% Group-III	155.59±3.08*
Ointment extract 10% Group-IV	221.75±2.47*

\*p<0.001 compared to control. Values are mean±SEM (n=6). *C. Africana*: *Centaurea africana*, SEM: Standard error of mean

The time of the epithelization was also found to be significantly shorter in animals treated with ointments containing the methanolic extract. It must be noted that the epithelization involves the proliferation and migration of epithelial cells across the wound bed [27]. Hence, the shorter epithelization time can be due to a facilitated proliferation of epithelial cells and/or to an increase of the viability of epithelial cells [28]. The hydroxyproline was used as a biochemical marker of collagen tissue. The rate of the increase of the hydroxyproline content of the treated groups has been significantly high compared to the control group, which indicates an increase in collagen synthesis [29]. Histopathological examination of wounds showed a more elaborate granulation tissue with a regeneration of collagen, an increase in vascularization, and an abundance of the fibroblasts in the wound area of the treated animals compared to the control ones [30]. In the study of the incision wound, the increase in tensile strength of treated wounds may be attributed to an increase in collagen concentration which is a constituent of growing cell and of the stabilization of the fibers [31]. Collagen is a major protein of the extracellular matrix and is the component that ultimately contributes to wound strength. This ability of plants to accelerate wound healing has also been observed on other plants [32,33]. The use of the ointment against acute inflammation (ear edema induced by croton oil) was also studied. This investigation showed good anti-inflammatory activity similar to that obtained with the reference product diclofenac gel. The application of croton oil can induce a significant inflammatory response, characterized by the apparition of edema, neutrophil infiltration, a prostaglandin production, and an increase in vascular permeability [34]. The anti-inflammatory compounds can act as effective agents for wound healing [35,36]. This investigation confirms the powerful healing and local anti-inflammatory properties of the methanolic extract of the roots of *C. africana* comparison to the respective reference product.

## CONCLUSION

The data generated from the present study indicate that the topical application of a methanolic extract of the roots of *C. africana* possesses the wound healing and anti-inflammatory activity. These results suggest that the traditional use of this plant is mostly justified. Further researches are needed to find the bioactive chemical constituents existing in the roots of *C. africana* responsible for these pharmacological activities.

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