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ANTIINFLAMMATORY AND WOUND HEALING ACTIVITY OF *HIPPEASTRUM PUNICEUM* BULB EXTRACT

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ABSTRACT

The present study was undertaken to evaluate the folkloric claim of *H.puniceum* bulbs for its anti inflammatory and wound healing activity. Aqueous extract of *H.puniceum* bulbs was prepared and used for the study. *In vitro* anti-inflammatory activity was evaluated by protein denaturation method and proteinase activity inhibition method using Diclofenac 100-500µg/ml as the positive control. An ointment prepared using the aqueous extract of *H.puniceum* bulbs was used for the *in vivo* studies. *In vivo* anti-inflammatory activity was evaluated by the carrageenan induced rat paw oedema model using Diclofenac ointment as the positive control. Wound healing activity was studied by the excision wound model using Betadine ointment as the positive control. The test extract exhibited significant anti-inflammatory activity *in vitro* and *in vivo*. The extract was also found to produce significant wound healing activity compared to the control. The results of the study underline the folkloric claim of *H.puniceum* in wound healing and reducing inflammation.

KEYWORDS: H. Puniceum bulbs, Antiinflammatory Activity, Wound Healing Activity, Betadine, Diclofenac.

INTRODUCTION

Mankind has been using herbs and herbal products for combating diseases since time immemorial. India has a rich tradition of herbal medicine as evident from Ayurveda, which could not have flourished for two thousand years without a scientific basis.[1] Plant based

pharmacological therapies are relevant as most of them are time tested and are proved to be safe and effective on large population.[2] As the demand of herbal medicine is increasing in both developing and developed countries, extensive research works had been conducted using plants but the therapeutic utility of ornamental plants had not been explored much. *Hippeastrum puniceum* is a widely seen ornamental plant whose bulbs were traditionally used in curing piles, swellings, tumours and various inflammatory disorders like asthma.[3]

It is a bulbous perennial ornamental plant, grown worldwide, but native to South America. The plant belongs to family Amaryllidaceae and is reported to contain alkaloids, terpenoids, carbohydrates, flavonoids and phenolics.[4] Pharmacognostic and physicochemical evaluation was conducted on its leaves.[5] Very few activity studies have been conducted on this plant and there are no reports on its anti-inflammatory and wound healing potential. 3-O-Narcissidine, an alkaloid isolated from this plant was reported to have antifeedant property [6]. NSAIDs like Ibuprofen, Diclofenac and Mefenamic acid have been ruling the market for decades in the treatment of inflammation. These drugs, although highly effective are associated with many potential side effects such as renal and hepatic damage. Therefore, it is high time for the scientific community to think of and search for a safer alternative such as a herbal remedy for inflammatory disorders.[7]Although herbs cannot completely replace the synthetic drugs in treating chronic ailments, they can be used in combination or be substituted wherever possible so as to reduce the potential adverse effects of synthetic medicine.

The plant *Hippeastrum puniceum* is well known for treating wounds and swellings and associated illnesses and the aqueous extract of its bulbs is traditionally used. Hence, the present study was undertaken to scientifically prove the claimed therapeutic utility of this widely seen ornamental plant.

MATERIALS AND METHODS

Drugs and chemicals: Diclofenac sodium (Sun pharma Ltd), Bovine serum albumin (Nice chemicals Ltd), Trypsin (Nice chemicals Ltd), Carrageenan (Nice chemicals Ltd), Betadine ointment (Win medicare pvt Ltd).

Plant material and Preparation of aqueous extract: The plant *Hippeastrum puniceum* was collected locally from Ettumanoor, Kerala. No special permission was sought for the plant collection as it is not an endangered species and as it is seen commonly alongside the village roads in Kerala. The plant material was identified and authenticated at Department of Botany,

St. Thomas College, Pala and preserved at University College of Pharmacy, Ettumanoor. The fresh bulbs were sliced in to small pieces and subjected to hot aqueous extraction for 6 h. The viscous solution thus obtained was filtered through a muslin cloth and concentrated at 60 ^{0}C. The dried extract was then stored in an air tight container until used. Preliminary phytochemical evaluation was conducted to generate an idea on the chemical profile of the aqueous extract. All experiments in this study were performed at University college of Pharmacy, Cheruvandoor, Kerala, India.

Animal experiments and ethical approval: Wistar albino rats (100-200 g) were obtained from small animal breeding station, Mannuthy, Kerala and acclimatized to the laboratory conditions for 2 weeks. They were housed in ventilated cages and fed with a pelleted diet and water *ad libitum*. The room temperature and relative humidity was maintained at 22 °C ± 3 °C and 30% – 70% respectively, with 12 h light and dark cycle. All the animal experiments were done in accordance with CPCSEA guidelines, after approval from Institutional animal ethical committee (012/MPH/UCP/CVR/27).

***In vitro* anti-inflammatory activity studies**

Inhibition of protein denaturation[8]

Diclofenac sodium 100-500 µg/ml in distilled water was used as standard (positive control) for the method. Sample solution was prepared by dissolving 1g of aqueous extract of *H.puniceum* bulbs in distilled water and solutions of 100-500 µg/ml concentrations were prepared.

The reaction mixtures consisted of 0.45 ml bovine serum albumin and 0.05 ml of *H.puniceum* extract. p^H was adjusted at 6.3 using 1 N HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3 min. After cooling, 2.5 ml phosphate buffer saline (pH 6.3) was added. Turbidity was measured spectrophotometrically at 660 nm. For control, 0.05 ml distilled water was used instead of extracts while standard contained 0.05 ml of diclofenac sodium. The percentage inhibition of protein denaturation was calculated.

Proteinase Inhibitory Action[8]

100-500 µg/ml of diclofenac sodium in distilled water was used as standard. Sample solution was prepared by dissolving 1g of aqueous extract of *H.puniceum* bulbs in distilled water and solutions of 100-500 µg/ml concentrations were prepared.

The reaction mixtures contained 0.06 mg trypsin in 1 ml of 25 mM tris HCl buffer (pH 7.4). 1 ml aqueous extract of *H.puniceum* was used as test and diclofenac as standard. The mixtures were incubated at 37 °C for 5 min. Then 1 ml of 0.8% (w/v) casein was added and incubated for 20 min. 2 ml of 70% perchloric acid was added and the suspension was centrifuged. Absorbance of the supernatant was read at 280 nm against buffer as blank and the percentage inhibition was calculated.

Preparation of ointments for *in vivo* anti-inflammatory studies[9]

The water soluble ointment base was formulated as follows. PEG 400 and PEG 4000 were melted in a china dish. The mixture was warmed at 65 ^{0}C and removed from the water bath. The herbal extract was added, stirred thoroughly until congealed and named HP ointment. Diclofenac ointment and drug free ointment was also prepared (only ointment base) in a similar manner. The prepared ointments were then evaluated for homogeneity, p^H, stability, extrudability, spreadability and skin irritation.

***In vivo* anti-inflammatory studies**

Acute toxicity study of the herbal ointment[10]

Healthy young adult wistar albino rats were acclimatized to laboratory conditions for 1 week prior to the test. Before the test, animals were randomized and assigned to the treatment groups. Approximately 24 h before the test, animals were depilated on the dorsal area of the trunk. NLT 10% of body surface area was cleared for the application of the test substance. Three albino rats weighing 100-200 g were selected and the animals were applied 2 g/kg dose topically to 10% of dorsal body surface area of formulated ointment (OECD guidelines no.402). The animals were critically observed for clinical symptoms, behavioural changes and mortality up to 72 h period and then up to a period of 14 days.

Carrageenan induced rat paw oedema model[11,12]

The animals were grouped in to test, positive control and negative control. In all the groups, inflammation was induced by a 0.1 ml injection of 1% W/V suspension of carrageenan in saline to the planar surface of right hind paw. In the test group, the formulated HP ointment was applied to the planar surface of the hind paw by gently rubbing 50 times with the index finger 60 min prior and after to carrageenan administration. Similarly, diclofenac ointment was applied to the animals in the positive control and ointment base was applied to the negative control. The change in the inflammatory reaction in various groups was measured

using plethysmometer at various time intervals (0, 1, 2, 3 & 4 hr) and compared with that in the negative control.

% inhibition of paw edema = $(Vt\text{-}V_0)$ control - $(Vt\text{-}V_0)$ treated/$(Vt\text{-}V_0)$ control

Vt is the rat paw volume at time t, V_0 is the initial rat paw volume (before carageenan injection), $(Vt\text{-}V_0)_{control}$ is edema produced in the negative control group and $(Vt\text{-}V_0)_{treated}$ is oedema produced in treatment groups (test and positive control).

***In vivo* wound healing activity study**

Excision Wound Model[13,14]: The animals were randomly divided into 3 groups of six each (test, positive control and negative control) and were anesthetized under light ether anesthesia, prior to the creation of wounds. The animals were shaven at the dorsal side and the anticipated area of wound to be created was outlined with methylene blue using a circular stainless steel stencil. A circular excision wound of about 500 mm^2 area and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. The animals of the test group and positive control were treated with HP ointment and diclofenac ointment respectively while the negative control animals were treated with ointment base. The ointments were topically applied once a day, starting from the day of operation, till complete epithelisation. The wounds were traced on mm^2 graph papers on days 0, 3, 6, 9, 12, 15 and 18 and thereafter on alternate days until healing was complete. The period of epithelisation was calculated as the number of days required for falling of dead tissue remnants of the wound without any residual raw wound. Percentage of wound closure was calculated by taking the initial wound as 100 %.

$$\text{Percentage wound closure} = \frac{\text{Initial wound size} - \text{Final wound size}}{\text{Initial wound size}} \times 100$$

Statistical analysis

The statistical analysis was performed by one-way ANOVA followed by Dennett's multiple comparison tests. The results were expressed as Mean ± S.E.M.

RESULTS AND DISCUSSION

Preparation of aqueous extract of *Hippeastrum puniceum* bulbs

The dry extract obtained after hot maceration of *Hippeastrum puniceum* bulbs was a pinkish white non sticky mass. The percentage yield was 3.12% w/w. This dry extract was used for the *in vitro* anti-inflammatory activity studies and for the formulation of HP ointment. The

extract was found to contain alkaloids, carbohydrates, mucilages, flavonoids, terpenoids, tannins and saponins.

In vitro anti-inflammatory activity

Inhibition of protein denaturation

The *in vitro* anti inflammatory activity of the aqueous extract of *Hippeastrum puniceum* bulbs was calculated and compared with the positive control, diclofenac sodium. The test extract was found to have a dose-dependent anti-inflammatory action *in vitro* and the inhibition recorded at the highest dose was 75.32 ± 0.60%. The results are shown in figure 1.

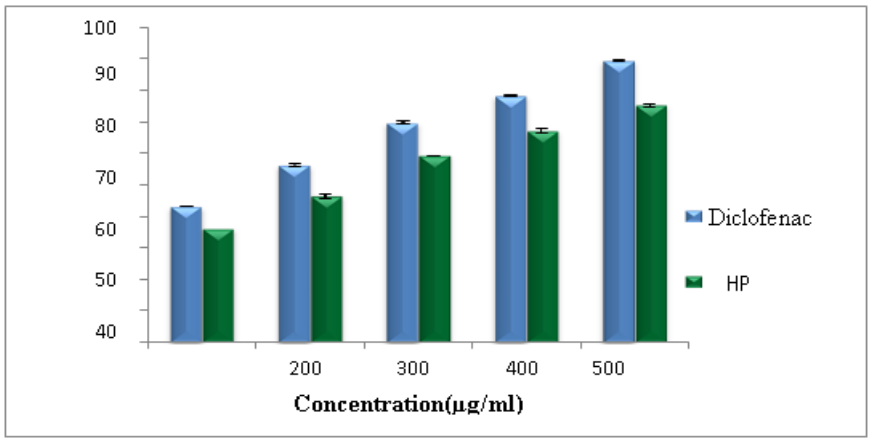

Figure 1. Comparison of percentage inhibition of protein denaturation

Proteinase inhibitory action

The *in vitro* anti-inflammatory activity of aqueous herbal extract using proteinase inhibition method was calculated and compared with standard diclofenac sodium. The test extract was found to have a dose-dependent anti-inflammatory action *in vitro* and 70.95 ± 0.41% inhibition was achieved at the highest dose tested. The results are shown in figure 2.

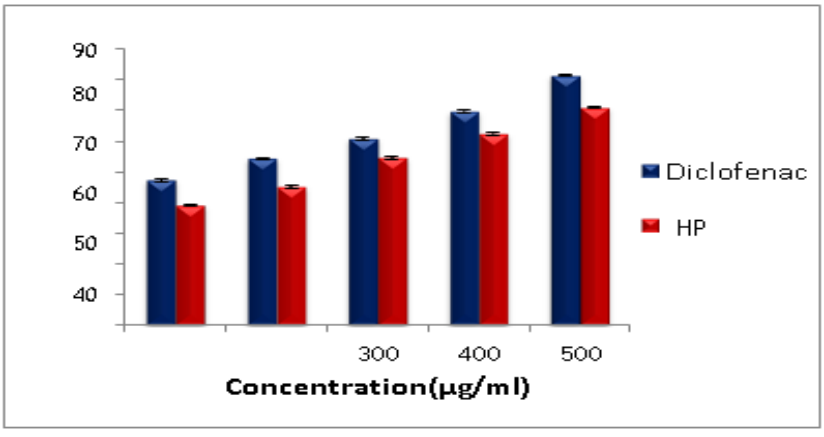

Figure. 2. Comparison of Percentage inhibition of proteinase activity.

Preparation and evaluation of ointments

Ointments for test group, positive control and negative control were prepared and subjected to preliminary quality control analysis. Skin irritation studies revealed that the above herbal ointment did not produce any skin irritation reaction, i.e., erythema and oedema after topical application. The quality analysis of ointments performed 3 months after the formulation revealed that the ointments satisfactorily maintained their properties with respect to colour, odour, p^H, spreadability and extrudability.

***In vivo* anti-inflammatory activity studies**

Acute toxicity study as per OECD guidelines 402

Acute toxicity study of the prepared herbal ointment was done using male wistar albino rats of 120-180 g. The HP ointment was applied on the dorsal surface of the animal and was observed for signs of toxicity. There were no mortality and no signs of toxicity. The parameters like body weight, food and water intake remained within the normal range throughout the period of study (14 days) indicating that HP ointment had no acute toxicity.

Evaluation of anti inflammatory activity by carrageenan induced rat paw edema model

The anti inflammatory activity of the prepared HP ointment was compared with that of formulated diclofenac ointment. Paw edema in all the groups was measured with the help of a Plethysmograph. The maximum paw volume was produced at the 2nd hour in all the groups and the edema was found to decrease thereafter. The reduction of rat paw edema by HP ointment and diclofenac ointment was highly significant from the 2nd hour to the 4th hour when compared to negative control ($p < 0.001$). (Table 1).

Table. 1. Paw volumes and percentage inhibition of paw oedema in various groups

Treatment	Paw volume in ml at hour					% inhibition of paw oedema			
	0	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Negative control	0.90 ± 0.002	1.17 ± 0.006	1.64 ± 0.007	1.54 ± 0.008	1.42 ± 0.006	-	-	-	-
Diclofenac ointment	0.96 ± 0.005	** 1.13 ± 0.007	*** 1.44 ± 0.005	*** 1.27 ± 0.004	*** 1.15 ± 0.005	37.0	37.8	54.6	67.3
HP ointment	0.95 ± 0.003	* 1.14 ± 0.010	*** 1.45 ±0.009	*** 1.28 ± 0.005	*** 1.15 ± 0.007	29.6	32.4	48.4	61.5

n=6, Values are Mean ± SEM. *p< 0.05,**p< 0.01 , ***p < 0.001

The reduction in paw edema brought about by HP ointment was comparable to that produced by the diclofenac ointment. The inhibition of paw edema by HP ointment was 61.5% at the 4th hour. The percentage reduction of paw edema in test group and positive control are tabulated in Table 1.

Evaluation of wound healing activity by excision wound model

The wound areas (mm^2) in various groups on days 0, 3, 6, 9, 12, 15, 18 are given in table 2 and percentage wound closure in various groups are given in figure 3. It is clear from the table 2 that there is significant reduction of wound area in the test group from the 3rd day ($p < 0.05$) and the wound area reduction is highly significant from the 6th day till the 18th day ($p < 0.001$). It is noteworthy that the potential of HP ointment in reducing the wound area is comparable to the positive control (Table 2).

Table. 2. Wound areas in various group from day 0-18.

Groups	Wound areas (mm2)						
	0th	3rd	6th	9th	12th	15th	18th
Negative control	507.5 ± 2.39	481.2 ± 3.66	404.5 ± 1.89	357.3 ± 2.85	266.2 ± 1.42	186.3 ± 2.76	75.17 ± 1.64
HP ointment	505.7 ± 2.61	429.3 ± 3.32*	322.8 ± 3.03***	206.2 ± 2.39***	95.83 ± 2.87***	14.83 ± 1.80***	1.17 ± 0.48***
Betadine ointment	506.7 ± 2.60	402.0 ± 2.07**	308.3 ± 1.98***	197.8 ± 2.40***	87.17 ± 2.07***	8.50 ± 0.88***	0.21 ± 0.17***

n = 6, values are Mean ± S.E.M. *p < 0.05, **p< 0.01, ***p < 0.001

The results indicate that the wound closure potential of HP ointment was highly significant ($p < 0.001$) and is more or less equal to Betadine ointment (Figure 3).

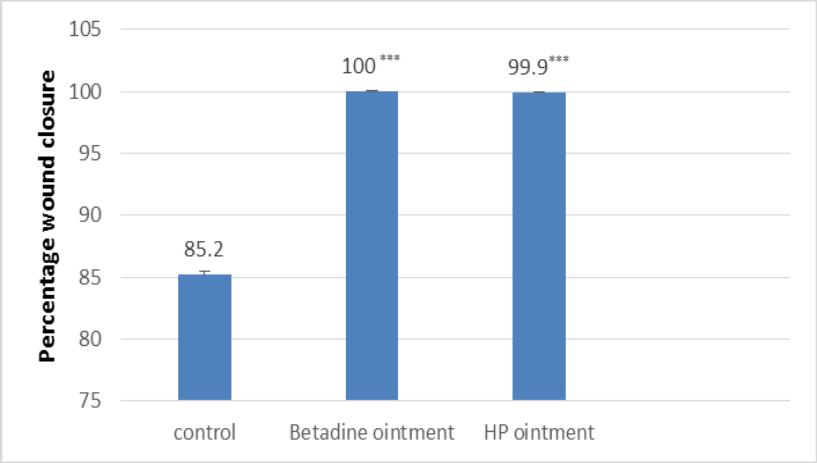

Figure. 3. Percentage wound closure in various groups.

n = 6, values are Mean ± S.E.M. *p < 0.05, **p< 0.01, ***p< 0.001

The epithelisation in the group treated with HP ointment was highly significant and this activity was found to be comparable to the positive control ($p < 0.001$) (Table 3).

Table. 3. Period of epithelisation in various groups.

Sl. No	Sample	Mean period of epithelisation (no. of days)
1.	Negative control	26.33 ± 1.26
2.	Betadine ointment	13.55 ± 1.32***
3.	HP ointment	16.33 ± 1.19***

n = 6, the results were expressed as Mean ± S.E.M to show differences in groups. The differences are considered significant when *P < 0.05, **P< 0.01, ***P < 0.001.

The data corresponding to the reduction in wound area, percentage wound closure and period of epithelisation reveal that HP ointment has significant potential for wound healing as further supported by figures 4-7.

Figure. 4-7. Wound healing in various groups.

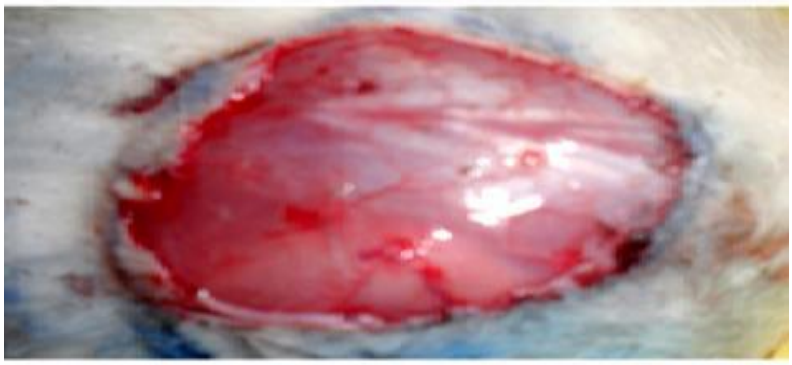

Figure. 4. Excision wound Day 1.

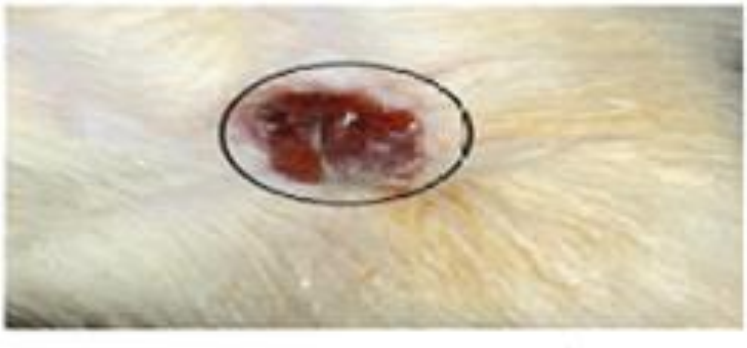

Figure. 5. Negative control Day 18.

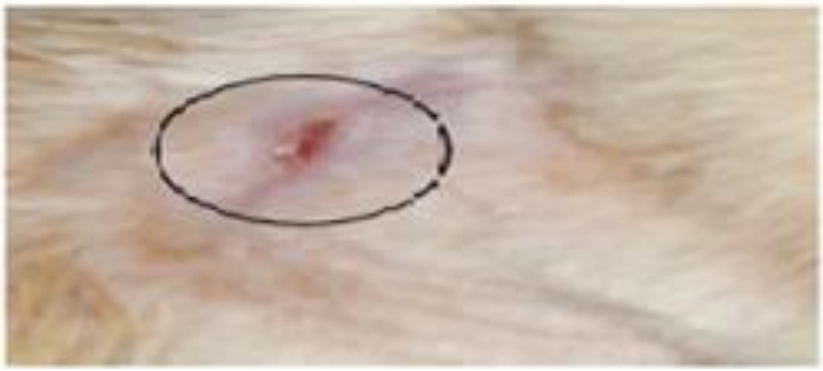

Figure. 6. HP ointment Day 18.

Figure. 7. Positive control Day 18.

Inflammation and wound healing are the tissue processes that may be acute or chronic. Usually, synthetic drugs are employed for the treatment of inflammation and antibiotics are used for wound healing.[15] When inflammation is treated on a chronic basis, the adverse effects and toxicity of synthetic drugs becomes a concern just as microbial resistance in case of improper antibiotic use.[16] There are many traditional and folkloric remedies for inflammation which are safe and effective. Such remedies should be brought in to light and be validated scientifically so as to substitute the synthetic drugs wherever possible. A traditional anti-inflammatory and wound healing remedy, the aqueous extract of *Hippeastrum puniceum* bulbs was evaluated in the present research to prove its folkloric claim.

Inflammation is a condition deriving from tissue response to trauma or pathogenic agents. It is a defensive way of response by an organism/tissue to remove the injuring stimuli, such as pathogens, damaged cells or irritants.[17] The inflammatory phase is the first and essential stage in the wound healing process and so the ability to reduce inflammation is an important criterion for a wound healing agent.[18] Inflammation is always associated with tissue damage through the production and induction of proteolytic enzymes and denaturation of proteins,

thereby leading to a delay in initiation of the repair phase.[19] The antiinflammatory potential of aqueous extract of *Hippeastrum puniceum* bulbs was studied both *in vitro* and *in vivo* and was found to have very significant activity. This activity may be attributed to the presence of flavonoids and phenolics in the bulbs which are well known antioxidants.[20] The high degree of wound healing exhibited by the bulb extract may be partly due to the presence of large amount of mucilage, which imparts a soothening action on the wound.[21] In addition, the high amount of mucilage can also provide protection from microbial growth and aids in healing.[22] However, detailed evaluation is required to identify and isolate the chemical entity responsible for the anti-inflammatory and wound healing potential of this plant.

CONCLUSION

The results of the study showed that aqueous extract of the bulbs of *Hippeastrum puniceum* possessed anti-inflammatory and wound healing properties as exhibited by the in vitro and in vivo studies. This proves its therapeutic utility and justifies its use in folkloric medicine for the treatment of inflammation and wound healing. Further research is required to identify and isolate the active compound responsible for the antiinflammatory and wound healing action. Comprehensive chemical and pharmacological investigation should be carried out on the aqueous extract and active compound to elucidate the mechanism of action.

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Conflicts of interest

There are no conflicts of interest

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