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**TOPICAL APPLICATION OF ECHINOPHORA PLATYLOBA HYDROETHANOLIC
LEAF EXTRACT, ENHANCED HEALING OF OPEN EXCISION WOUND IN RATS**

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ABSTRACT

In the present study, we evaluate the ethno pharmacological usage of the root parts of Turmeric extract, using in vivo pharmacological experimental models.

In vivo excision wound models were used in order to assess the wound healing effects (wound contraction and histopathological assay) of the hydroethanolic extracts of the plants. Skin samples were also evaluated. Forty five rats were divided into four groups of eight rats each. Group 1 (negative control), group 2, placebo (as the reference standard group) and groups 3, 4 and 5 (experimental) applied respectively with the 1g, 2g and 3g of powder extract mixed with base formulation until complete epithelialization. Wound area was monitored on days 3, 5, 9, 11, 13, 15 and 17 with graph paper and histological evaluation was carried out on the samples on days 3, 7, 14 and 21.

The results show that all ointment formulation prepared with hydroethanolic extract of *E. platyloba*, especially group 2%, more effective in stimulating the enclosure of wounds, increased fibroplasia, re-epithelialization and collagen disposition in treatment groups compared to the control groups, and remarkable promote wound healing activity in rat as an in vivo experimental wound models.

All therapeutic doses of *Echinophora platyloba* hydro alcoholic extract played a preeminent role in the delayed wound healing activities compared to control group. This herbal formula accelerated wound healing and 2% topical ointment did well.

Keywords: Hydro Alcoholic Extract, Echinophora Platyloba, Circular Excision Wound, Wistar Rats

INTRODUCTION

Wide spread of using plants which have a potential of treating disease in the favor of medical approach is undeniable. On the other hand, increasing the public interest in using folk medicine and herbal cures have been observed all over the world specially developing countries. High price of synthetic medications, side effects of them, availability of herbal medications and etc. persuade people to take these plants to cure their illnesses. Sometimes sanctions are another item which forces people to use alternative medications like herbal treatments.

Echinophora platyloba (*E. platyloba*) is a plant from the flora of Iran and used in traditional medicine [1]. *Echinophora* belongs to the family of Apiaceous or Umbelliferae which is represented in the flora of eastern Asia and Mediterranean region. These families have 10 different species which *E. orientalis*, *E. sibthorpiana*, *E. cinerea*, and *E. platyloba* them are native for Iran [2]. There are lots of reports, that *echinophora* various species due to having chemical constituents, including phenolic components, Coumarins,

flavonoids, alkamides, monoterpens and polysaccharides,(3, 4) have been good biological activities, including anti-fungal effect especially against *Candida albicans*, [1, 5] preventing symptoms of premenstrual syndrome, [6] anti-oxidant activity, [7] antispasmodic effects in the rat ileum, [8] and the use of topical administration, have been anti-inflammatory [9, 10] and wound healing properties [11, 12].

This study was conducted in order to evaluate the non-infected excisional wound healing activity of *E. platyloba* hydro alcoholic extract on induced wounds on rat. Since this folk medicinal plant has a lot of applications, the aim of this research was to provide a scientific demonstration for its folk usage on ability of rapid repair of injuries, if there was any.

MATERIALS AND METHODS

Plant material and extract preparation

Fresh leaves of *Echinophora platyloba* were collected locally during July 2011 in the region of Tuyserkan city (latitude: 34 32' 53", longitude: 48 26' 49"); and identified by the Department of Botany Sciences, the Hamadan

researches Agricultural and Natural resources center. The plant material was dried naturally on laboratory benches at room temperature (23-24°C) for six days until crisp, and powdered in an electric blender. Then 250 g of the Mentioned plant powder was suspended in 500 ml of hydroethanolic solution for 96 h at room temperature. The mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No 1). The filtrate was placed in an oven to dry at 40 °C. The clear residue obtained was used for the study. The extracts were kept at -15 °C until it was used in the experiment.

Biological activity test

Animals and study design

Healthy white Wistar male rats weighing approximately 200 g and 9 weeks of age were included into the present study. Sixteen healthy white Wistar rats of either sex were used for acute toxicity studies. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages (50 × 40 × 20 cm) with an ambient temperature of 23 ± 3° C, stable air humidity, and a natural day/night cycle. The animals were handled on a regular daily basis for 2 weeks prior to the study in order to acclimatize them with testing area and experiments. This could minimize anxiety related testing inaccuracies. The rats had free

access to standard rodent laboratory food and tap water. The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain (**Zimmermann, 1983**). The University Research Council approved all experiments.

Acute toxicity study

In order to find a safe dose for *Echinophora platyloba* L., acute toxicity study was conducted. Sixteen healthy white Wistar rats of either sex, eight males and eight females, approximately 200 g and 6 to 8 weeks of age were randomly divided into 4 groups of 4 animals each: Control, Placebo, 5% and 10% of *Echinophora platyloba* L. groups. The animals were under surveillance for 30 min 2, 4, 24 and 48h after the administration for the onset of clinical or toxicological symptoms. Mortality rate was recorded in a period of two-week, if any. The animals were euthanized on day 14 post-test. Hematological, serum biochemical and histological (liver and kidney) parameters were determined based on the standard methods described by others (**Bergmeyer et al., 1980; Souba et al., 1999**).

Formulation of topical wound application forms

Four variants of the topical application ointment were prepared. All the variants

consisted base formulation comprising Eucerin (25%) and Vaseline (75%) in about 1:3 proportions.

A surgical wound was created, all rats randomly were labeled by none toxic color and divided into five groups. Two groups served as Controls: Group 1 did not receive any administration and Group 2, Placebo; animals were applied with the base formulation. Groups 3, 4 and 5 were applied with 1, 3 and 5g of *Echinophora platyloba* L. hydroethanolic extract mixed with base formulation (EPO), respectively (TrivellatoGrassi *et al.*, 2013). The ointments were topically applied once a day, starting from the day of operation, on the wound area until the wound healed completely. All rats were monitored for any wound fluid or any evidence of infection or other abnormalities, until complete epithelialization.

Wound healing models

Circular Excision Wound Model

Animals were anesthetized by intraperitoneal administration of ketamine 5%, 90mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). The fur was prepared aseptically and the predetermined area was marked on the back of animals. Each rat was fixed on the surgery table in ventral posture. Following

surgical preparation a circular surgical full thickness wound was made, 314 mm² diameter, on the anterior-dorsal side of each rat. Wound contraction percentage and wound closure time were used to assess wound-healing property.

The wound area was measured by immediate placing of a transparent paper over the wound and tracing it out, area of this impression was calculated using the graph sheet. The wound healing percentage was calculated by Walker formula after measuring the wound size (Walker & Mason, 1968). The percentage of wound healing was computed at the beginning of experiments and on days 3, 6, 9, 12, 15, 18, and 21 days post-test.

Percentage of wound size = $\frac{\text{Wound area on day X}}{\text{Wound area on day zero}} \times 100$

Percentage of wound healing = $100 - \text{Percentage of wound size}$

Incision wound model

Animals were randomly divided into five experimental groups of six animals each: Control, Placebo, 1%, 3% and 5% groups. All animals of experimental groups were anesthetized with the same way mentioned above and a of 4-cm length incision was made through the skin and cutaneous muscle at a distance about 1.5 cm from the middle on right side of the depilated back. The wound was closed at 0.5 cm intervals using 3/0 nylon

(Dafilon, B/Braun, Germany). All the groups were treated the same as mentioned in the excision model. Ointments were applied once daily for 9 days. On day 9, sutures were removed and the tensile strength of healed wounds was measured on day 10 by Strongraph mechanical test frame (Toyoseiky Tensile Testing Unit, Model R3, Japan) (Derakhshanfar A *et al.*, 2010)

Tensile strength was calculated using the following formula:

Tensile strength = breaking strength (g) / cross sectional area of skin (mm²)

Histopathological Study

Animals were anesthetized with the same way mentioned above and specimens from skin were taken on 3, 6, 9, 12, 15, 18 and 21 days after surgery. Sample tissues, excised along with 1 to 2mm surrounding normal skin and in a depth of approximately 3mm, were pinned on a flat cork surface and fixed in neutral-buffered formalin 10%. Then the sample tissues were routinely processed, paraffin wax embedded, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) and Masson's trichrome stains and examined under light microscopy (Olympus CX31RBSF attached cameraman) to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Following factors such as Cellular

infiltration (the number of mononuclear cells, poly morphonuclear cells and Fibroblastic aggregation), angiogenesis (the number of blood vessels and capillary buds) were quantitatively evaluated in 5 per high power fields (HPFs) ($\times 400$). Acute hemorrhage, congestion, edema, epithelialization, collagen production and density were also evaluated qualitatively and calculated manually. They were analyzed in 5 per high power fields (HPFs) ($\times 100$) (Karayannopoulou *et al.*, 2011; Akkol *et al.*, 2011).

Statistical Analysis

Experimental results were expressed as means \pm SEM. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using one way ANOVA. Dunnett's test for pair-wise comparisons was used to examine the effect of time and treatments. Differences were considered significant when $P < 0.05$, $P < 0.01$.

RESULTS

Rate of Wound Healing

The data of percentage of wound contraction is shown in **Figure 1**. The wound sizes were measured on 3, 5, 9, 11, 13, 15 and 17 day of post-surgery. The extract showed a significant decrease in the rate of wound size beginning the seven day ($P < 0.05$). The sizes were

reduced more quickly in the treatment groups, especially in group EPO 2%, compared to the control group. At the last day of measurement the wounds in the treatment groups were approximately enclosed, while the control and placebo had rather large wounds.

Histologic evaluation

Following histopathological examination, the qualitative results of the sample tissue slides in 3rd, 7th, 14th and 21st days, which stained with haematoxylin – eosin and Masson's trichrome stains, evaluated, scored and presented in **Table 1**. Note, none of the wounds of the treatment groups, the infectious organisms were not detected.

DISCUSSION

Wound healing is a process of three complex physiological phases begins after the damage known as inflammatory, proliferative and remodeling phases [13]. Accurate occurrence of all these phases makes the healing process repair the wound as normal as it possible. Accelerating each of these phases will lead to enhancing the healing process by mean of time and physiological process.

There are lots of reports, that echinophora various species due to having chemical constituents, including phenolic components, Coumarins, flavonoids, alkamides, monoterpens and polysaccharides [3, 4]. There are many reports that phenolic

compounds alkamides, and flavonoids may have therapeutic effect such as antioxidant and anti-inflammatory properties [14, 15] and wound healing [16, 17]. Flavonoids whit increase of the viability of collagen fibrils by Inhibition of lipid peroxidation effect, cause to promote the wound healing process [18]. Furthermore, terpenoids with increased rate of epithelialization and wound contraction cause to promote the wound healing process [19].

Based on quantitative and qualitative histopathology findings of the wound edge with H&E and Masson's trichrome stains, was determined all treated group with *E. platyloba* ointment by comparison with a untreated and placebo group has created a more favorable result.

The early finding of neutrophils role indicates that they just omit the contaminations from the area; that this contamination causes delayed wound healing. In this study, the mean number of PMN cells, in treatment groups was lower than the control groups, with increased concentrations of plant extracts, the differences are greater (**Figure 2**).

Macrophages, after neutrophils are imported the wound site. This cells as main cells of inflammatory phase [20], with secrets a lot of key mediators including various growth factors and cytokines, which is cause the

migration, and increase fibroblasts and myofibroblasts into the wound site, which is to promote and facilitate collagen synthesis [21, 22]. Second dose of *E. platyloba* (2% EPEO) had a significantly increasing effect in the level of macrophages at day three (**Figures 3**). Low numbers of neutrophils and the high number of macrophages on the third day after the injury, in the treatment group, Signs of wound healing is accelerated.

As seen in this **Figure 4**, at the third day after wound creation, two doses of *E. platyloba* dosages (1% and 2%) showed a good capability to increase the neovascularization.

In the seventh and fourteenth days after wounding, the increased rate of fibroblasts number in treatment groups 2% and 3% EPEO (**Figure 5**). At the same time, begins the proliferation of fibroblasts into the wound site and the epithelial cells from the wound edges side to the center. These events prepare a surface for formation of a granulated and dense shape tissue which is so called granulation tissue [21, 22]. It plays a barrier

role to protect the vulnerable injured site from infectious microorganisms. Meanwhile the granulation tissue contracts the wound to enclose the injury [23, 24].

Collagens on the other hand, line in a parallel position and bring the edges to gather by contraction. So the real healing procedures will be accelerated by the increasing fibroblasts which produce collagens and epithelialization (**Figure 6**).

In the proliferative phase new cells divided from re-epithelialization refill the area from the margins toward the center of injury. **Figure 7** showed effect of *E. platyloba* L. hydroethanolic leaf extract on epithelial thickness.

Our results indicate elevation in both of the aforementioned factors, and as it was expected *E. Platyloba* had a very promising effect in acceleration of wound healing in time of process and the quality of it.

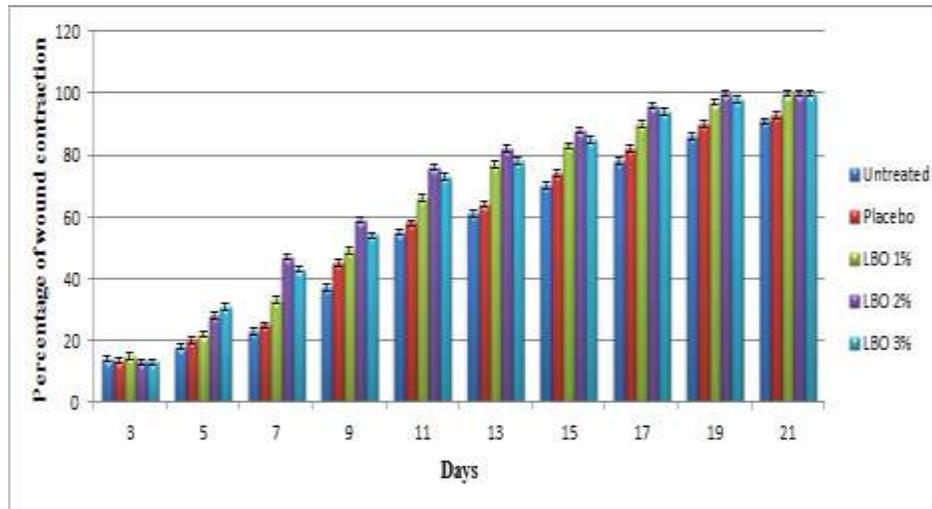


Figure 1: Effects of *Echinophora platyloba* hydroethanolic extract L. extract ointments on wound contraction (percentage of contraction)

NOTE: Valued are expressed as mean \pm SEM (standard error mean); n= 6 animals in each groups. * $P < 0.05$ vs Control

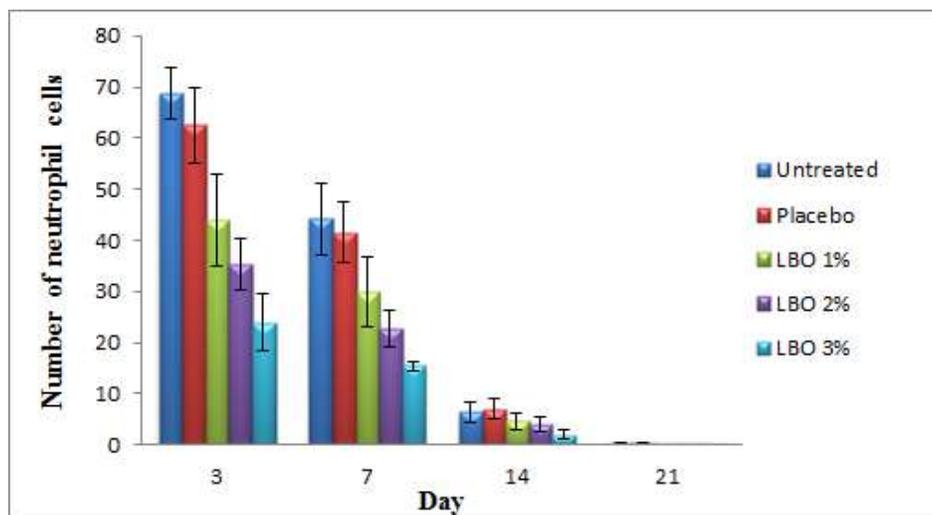


Figure 2: Effects of *E. platyloba* L. hydroethanolic leaf extract on measurement of number of polymorphonuclear cells infiltration (PMN) during 21 days

Valued are expressed as mean \pm SEM (standard error mean); n= 2 animals in each groups. * $P < 0.05$ vs Control

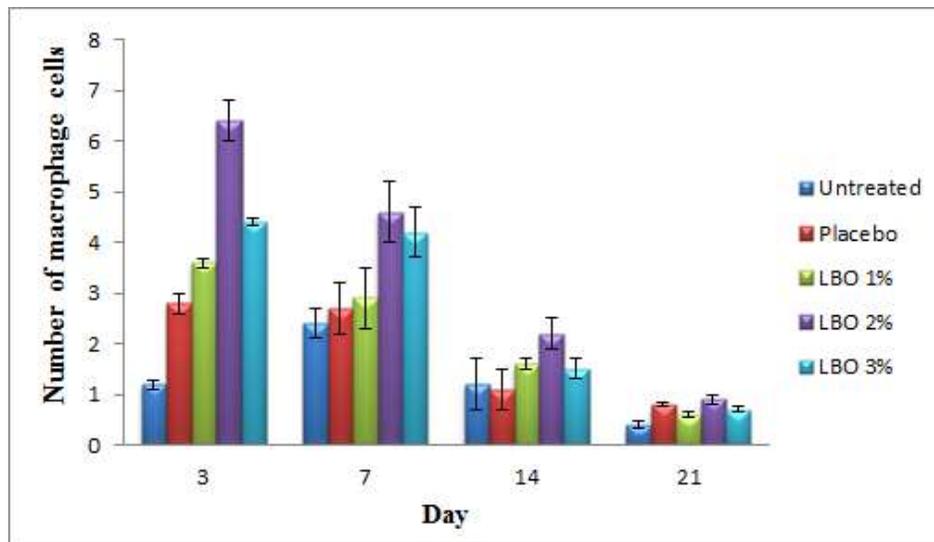


Figure 3: Effects of *E. platyloba* L. hydroethanolic leaf extract on measurement of number of mononuclear cell infiltration (MNC) during 21 days
 Valued are expressed as mean \pm SEM (standard error mean); n= 2 animals in each groups. * P <0.05 vs Control

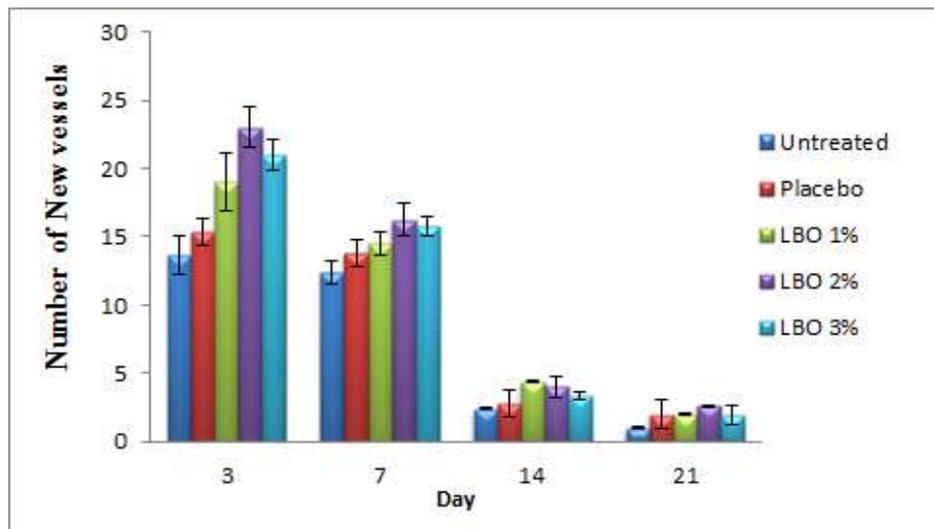


Figure 4: Effects of *E. platyloba* L. hydroethanolic leaf extract on measurement of new vessels number during entire experiment
 Valued are expressed as mean \pm SEM (standard error mean); n= 2 animals in each groups. * P <0.05 vs Control

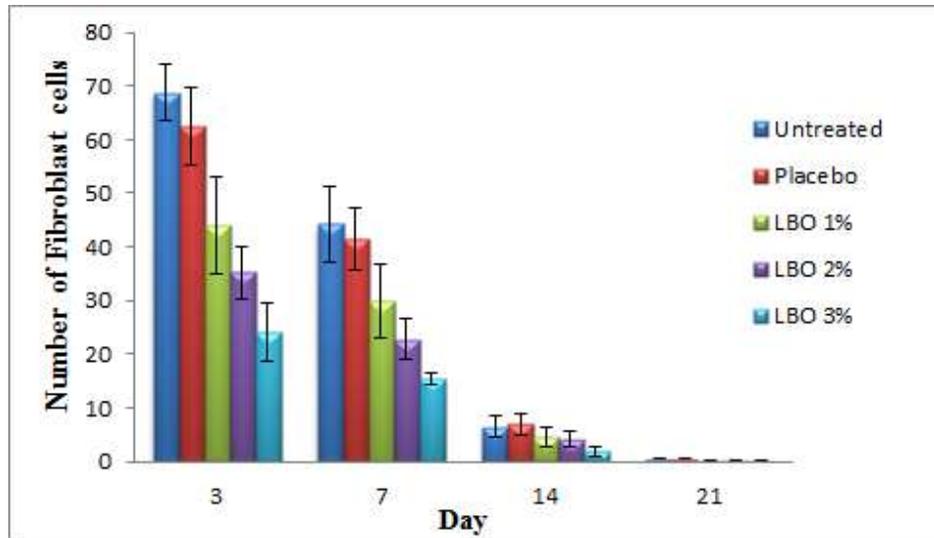


Figure 5 : Effects of *E. platyloba* L. hydroethanolic leaf extract on measurement of fibroblastes proliferation number during entier experiment

Valued are expressed as mean ± SEM (standard error mean); n= 2 animals in each groups. **P*<0.05 vs Control

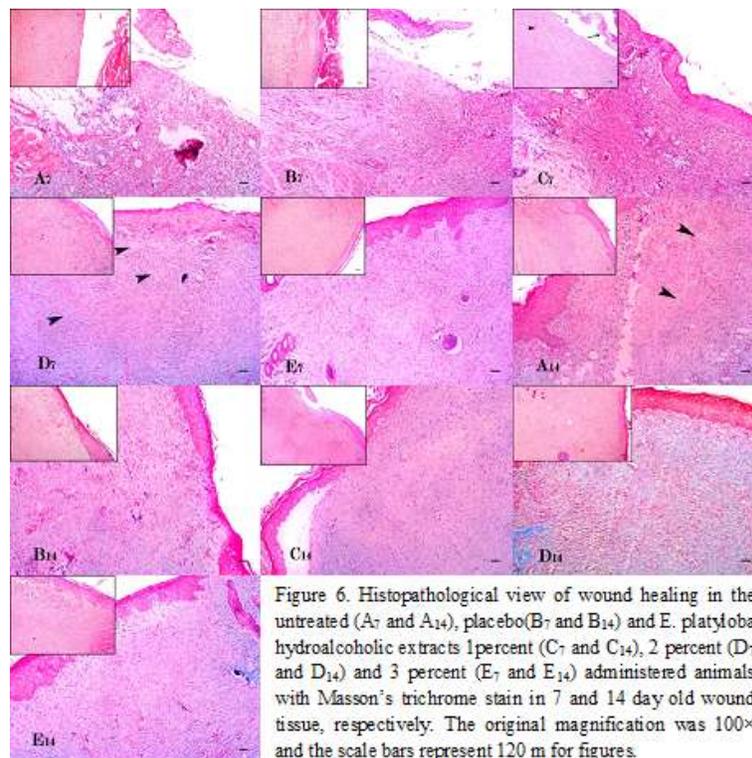


Figure 6. Histopathological view of wound healing in the untreated (A₇ and A₁₄), placebo(B₇ and B₁₄) and *E. platyloba* hydroalcoholic extracts 1percent (C₇ and C₁₄), 2 percent (D₇ and D₁₄) and 3 percent (E₇ and E₁₄) administered animals with Masson's trichrome stain in 7 and 14 day old wound tissue, respectively. The original magnification was 100× and the scale bars represent 120 m for figures.

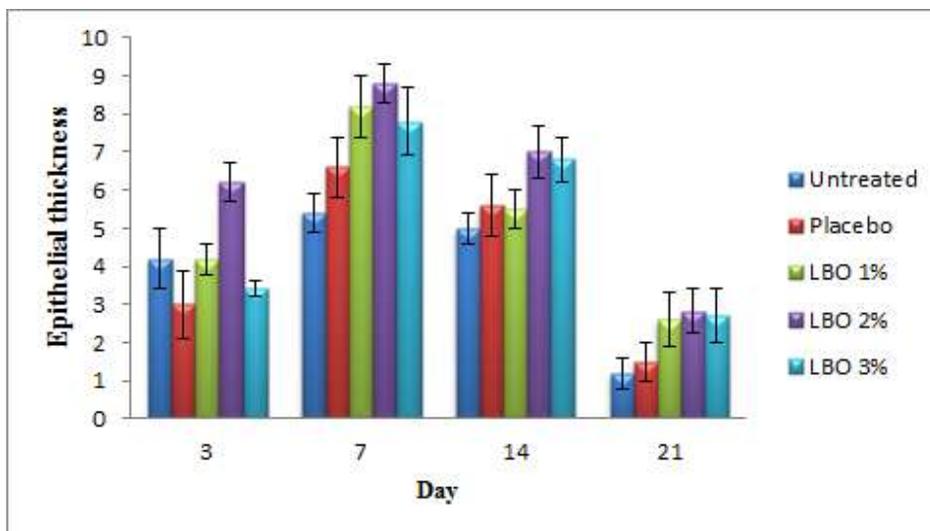


Figure 7 : Changes in mean ± SEM of Epithelial thickness score during the excision wound healing process. Values are expressed as mean ± SEM (standard error mean); n= 2 animals in each groups. *P<0.05 vs Control

Table 1: Histological evaluation of Echinophora platyloba (EPO) Extract on wound healing process in different groups of treatment in rat

Concentrations	Day	Ed score	Hem score	Con score	Fib score	Cp score
Negative control	3	++++	+++	++++	-/+	-
	7	++	++	++	+	+
	14	+	+	-	++	++
	21	+	+	-	+++	+++
Placebo	3	++++	++++	+++	-/+	-
	7	+++	+++	+++	+	+
	14	+	+	++	++	++
	21	+	-/+	+	+++	+++
Placebo +1gr E.platyloba	3	++++	++++	+++	+	-/+
	7	++	++	++	++	+/+++
	14	++	-/+	+	+++	++/++++
	21	+	-	+	++++	+++
Placebo +2gr E.platyloba	3	+++	++	+++	+	-/+
	7	+	-	++	++/++++	++
	14	-	-	+	+++ /++++	+++ /++++
	21	-	-	-/+	++++	++++
Placebo +3gr E.platyloba	3	+++	+++	++++	+	-/+
	7	++	+	+++	++	++
	14	+	+	++	+++ /++++	++ /+++
	21	+	-	+	++++	++++

Hematoxylin- eosin and Masson’s Trichrome staining sections were scored as absent (-), present (+), mild (++) , moderate (+++) and severe (++++) for Ed: edema; Hem: Hemorrhage score; Con: Congestion; Cp: Collagen production and Fib: Fibroblast cells

CONCLUSION

In conclusion, According to the results reported here, topical administration of hydro ethanol extract of *E. platyloba* in differential doses, especially ointment 2% compared to other doses and two control groups, remarkable promote wound healing activity in rat as an in vivo experimental wound models, and it may be suggested for treating various types of wounds in animal and human beings.

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