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OIL EXTRACT OF *LORANTHUS EUROPEUS* SEEDS PROMOTES WOUND HEALING

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ABSTRACT

The efficacy of oil extract of the seeds of *Loranthus europeus* on wound healing was investigated. After the preparation of the oil extract, the oil ointment of *L. europeus* was prepared. A preliminary biochemical analysis were carried out to find out the chemical contents of *L. europeus* oil extract. The study involved 18 male rabbits, they were divided equally and randomly into 3 groups depending on post wounding biopsy: (3^{rd},7^{th} and 14^{th}day post wounding). Two full thickness cut wounds were done on the both sides of shoulder regions(the left wound as control and the left as treated wound) of each group of animals. The treated and control wounds were treated continuously with (0.5mg) oil ointment and vasceline base respectively, for 14^{th} day twice a day. All wounds were evaluate macroscopically which included "measurement of contraction rate, daily wound contraction, hyperemia, exudation and scab formation." and microscopically for "neutrophil, macrophage infiltration, re-epithelization, fibroblast proliferation with collagen production and new blood capillary formation".

Both macroscopic and microscopic results showed the efficacy of *L europeus* seeds in promoting the healing process significantly as compared with control wounds ($P<0.05$). The oil extract treated wounds showed significant increase in hyperemia, exudation and scab formation, neutrophils and macrophages infiltration, fibroblast proliferation with collagen production and formation of new epithelium (re-epithelization), contraction rate and daily wound contraction at 3^{rd} day post wounding, as compared with control wounds, but these categories showed reduction at 7^{th} day except in macrophages, re-epithelization and fibroblast with collagen production which all showed significant increase at 7^{th} and 14^{th} days post wounding as compared with control wounds. The preliminary chemical analysis for oil extract showed the presence of Glycosides, Carbohydrates, Aldehydes & Ketones, Trifrepenoides groups, protein and Polysaccharides, while Alkaloides, Flavonoides and Saponins are absent.

INTRODUCTION

Wounds can be defined as any processes which lead to disruption of normal architecture of a tissue **(1)**. They may be: closed (e.g.: bruises, ruptures and sprains), or open (e.g.: abrasions, laceration, avulsions, hernias and excised or surgical wounds) **(2)**. Wounds can be classified according to the number of skin layers affected to: *Superficial wounds, Partial thickness wounds and Full thickness wounds*. **(3)**. Generally, healing starts immediately after damage has occurred, but the mechanism, speed of healing and the eventual nature of the regenerated tissue depend on the type of wound. There are three main phases of wound healing: Inflammatory phase, repair phase (which may be further subdivided into proliferation and organization) and maturation / regeneration phase (including contraction and remodeling) **(2,4-11)**.

Several field and laboratory studies explained that there are several plants which is used medically for treatment of skin wounds. *Loranthus europeus* characterize by their medical and economical importance which is used in several countries of world **(12).**

In Iraqi folk medicine *L. europeus* seeds were used for treating abscesses. it is claimed that the poultice causes maturation and acceleration the drain of the pus from it. However, the mechanism of action of these seed is unknown till now. Consequently, it is thought to be interesting to investigate the wound healing efficacy of oil extract of *L. europeus* seeds.

MATERIALS AND METHODS

Preparation of oil extract ointment: The seeds of *Loranthus europeus* had been bought from local market in Basrha Province/Iraq. Kept in polyethylene bags at room temperature until the use time. After cleaning, the seed were chopped were transferred to the thumble of soxhlet apparatus, extracted with (400 ml) petroleum ether (B.P 40-60 C^{o}, BDH, England) for 24 hrs. Then the solution was concentrated by rotary evaporator, (Puchi Rotavapor, RE) at $50C^{o}$, the final dryness was done by the evaporation of remnant solvent by leaving the residue in room temperature, the resultant was (31gm) viscous oil. The ointment was prepared with vaseline base. The ratio of oil extract to vaseline was 3:1 **(13).**

Preliminary chemical analysis for the extract: To determine the chemical groups in the oil extract, the following Chemical tests had been done: *carbohydrates test*: by use Molish reagent **(14),** *Flavonoides test*: by use maganessium turinges and Alcoholic potassium hydroxide solution (Alcoholic KOH(5n)**(15),** *Saponin test*: by use aqueous mercuric chloride (5%),($Hgcl_3$5%) **(16),** *Glycosides test*: by use Benedict's reagent **(15),** *Aldehydes and Keton's test*: by use 2, 4 Dinitro phenyl hydrazine reagent **(17),** *Alkaloides test*: by use Dragendroff reagent **(18),** *Tri terpenorides test*: by use sulfuric acid and chloroform. **(19),** *Protein's test*: by use Biuret reagent **(20)** and *Polysaccharides test*: by use Iodine reagent **(21).**

Animals and housing: The animals used in the present study were male domestic rabbits *(Lepus domestica)* of (4-5) months age, body weight (1000- 1250) grams, those rabbits were housed in metallic cages (100 ×40 ×193 cm). They were fed on alfa alfa and water *ad libitum.*

Experimental Design: Eighteen male rabbits divided randomly into three groups of six animals per each group according to the date of post wounding biopsy, those groups were: ***Group 1:*** (3rd -day post wounding), ***Group 2*** (7^{th} -day post wounding) and ***Group 3*** (14^{th} day post wounding). All rabbits were clipped and prepared for aseptic surgery. They were anesthetized with intramuscular administration (I.M) of 10mg / kg body weight xylazin hydrochloride (Rompun, Haverlock Hart, Shawnee, Ks.) and 50mg /kg body weight Ketamin hydrochloride (Ketanes, Areco. Fort Dodge, IA.). Then at the dorsal aspect (shoulders of the animal near the neck). a circular open wound (cut) was done including a full thickness of skin layers after determination the area with a marker with a known diameter 2cm. The treated wounds were on the right side, and the control were on the left side of the animal, then (0.5g) oil extract ointment had been applied on the right sided treated cut wounds, the left sided cuts (control wounds) were treated with (0.5g) vaseline base only. The ointment and vaseline were applied to the treated and control respectively. twice daily for 14 days.

A- Macroscopic evaluation: All wounds were examined at the determined intervals

***1-wound contraction*:** At the day of wounding (day 0), each wound site from individual rabbit was digitally photographed at the indicated time intervals. The degree of wound contraction (expressed as a percentage) was calculated using the following equation **(22)**:

$$\left[\frac{\left(A_{Dayo} - A_{Dayx}\right)}{A_{Dayo}}\right] \times 100$$

Where: **x** = 3, 7, 14 day post wounding, **A** =wound surface area.

2. Wound contraction per a day: The rate of wound contraction per a day is calculated by dividing the difference between mean diameter of wounds at operative day (0 day, 2cm) and the day of complete closure (or last experimental day, 14th day), on the number of days required for complete wound closure (or the number of experimental days, "14 days", if complete occlusion required longer time).

3-Hyperemia, exudation and scab formation: using the following scores:*0 represent non or absence, 1 represent mild, 2 represent moderate and 3 represent sever*

B-Microscopic evaluation: Under general anesthesia, both control and treated wounds were excised at the determined date. Then formalin fixed, paraffin impeded, haemotoxyline-eosin stained and histological sections were prepared. The prepared sections were evaluated for inflammatory cells, fibroblasts, capillary proliferation and re-epitheliazation, which is depending on the progression of new epithelium to cover the defect, using the following score: 0 represent no epithelium; 1 represent new epithelium covering up to 1/3 of the defect; 2 represent new epithelium covering up to 2/3 of the defect; 3 represent complete re-epitheiliazation.

Statistical analysis: The results were analyzed by one –way ANOVA test using SPSS (version 9.0) .All data are expressed as Mean ± SD., the difference between groups were considered significant at ($P<0.05$).

RESULTS

Preliminary chemical analysis for oil extract: The preliminary chemical analysis for oil extract showed the presence of Glycosides, Carbohydrates, Aldehydes & Ketones. Trifrepenoides groups, protein and Polysaccharides, while Alkaloides, Flavonoides and Saponins are absent.

Macroscopic Evaluation: The effect of oil ointment of *L. europeus* on the wound contraction rate is presented in Table (1). The wound contraction rate was significantly higher in treated group than in control group at all intervals ($P<0.05$). A complete wound closure was achieved at 11th -12th day post wounding in all treated wounds. While the control wounds failed to achieve a complete closure till the end of experiment at 14th day post wounding.

Table (2) showed the result of decreasing in wound diameter for both treated and control wounds. At 3rd- day post wounding, both treated and control wounds, showed non significant difference in decreasing the wound diameter. On 7th- day, there is a significant decrease in diameter of *L. europeus* oil extract treated wounds, and at 11th- day the diameter for treated wounds a significantly decrease ($P < 0.05$) and become (0 cm). The daily wound contraction in wounds treated *L. europeus* oil extract was(1.8 mm / day) , while in control wounds, was (1 mm / day) as shown in Table (3).

The results of the effect of *L. europeus* oil extract on different wound healing categories (hyperemia, exudation and scab formation) were explained in Table (4). On 3rd day post wounding, the treated wounds showed a significantly increased in all wound healing categories (i.e. hyperemia, exudation and scab formation) than in control wounds ($P< 0.05$). On 7th day post wounding, there was more reduction in severity of hyperemia and exudations in treated wounds than in control wounds ($P< 0.05$), while the scab still thicker in treated wounds. Both hyperemia and exudation had disappear completely in treated wounds by the day 14th and the scab became thinner with persistence of very mild exudation in control wounds.

Microscopic evaluation: The results of the effect of oil extract on histological elements of wound healing were showed in Table (5) and Figures (1-10).

On 3rd-day post wounding, the infiltration of neutrophils was significantly higher in treated than in control wounds (P< 0.05), figure (1), and (3). On 7th day, the neutrophil infiltration is significant higher in control than in treated wounds (P< 0.05), Figures (5,7). The infiltration became mild on 14th day in control wounds and disappeared completely in treated wounds, Figures (9,10).

The infiltration of macrophages was significantly higher in treated wound than in control wounds at 3rd and 7th day (P< 0.05), and their infiltration became more sever in both control and treated wounds at 14th-day. The fibro-vascular granulation tissue (new blood capillaries and proliferative fibroblast with collagen) started to appear on the 7th – day and became more obvious on 14th - day, the new capillaries and fibroblast proliferation was significantly higher in treated wounds (P< 0.05), Figures (1, 3, 7, 9, 10).

Through out the period of experiment, the progression of new epithelium to cover the wound area is significantly higher in treated wound than control wounds (P< 0.05), Figures (2, 4, 6, 8, 10).

Table (1):Effect of *L. europeus* oil extract on wound contraction rate (mean±SD),(n=6)

Groups	Wound contraction rate %		
	3rd day	7th day	14th day
Controlled(C)	12.7± 7.34	37.0 ± 9.61	87.8 ± 4.77
Treated (T)	*42.1 ± 9.96	*70.1 ± 6.73	*100.0 + .00

(*) Differences between (T&C) are significant at level (P< 0.05).

Table (2): The effect of *L. europeus* oil extract on mean diameter of wounds at different intervals, Mean±SD, (n=6).

Groups	Mean diameter at different in travels (cm).				
	0 day	3rd day	7th day	11th day	14th day
Control (C)	2 .0± 0.00	1.8±8.16	1.5±0.11	0.9±0.	0.6±1.47
Treated(T)	2.0± 0.00	1.5±0.13	*1.0±0.11	*0.0±0.0	*0.00±0.0

Number of animals = 18 rabbits, Mean ± SD

(*) Differences between (T&C) are significant at level (P< 0.05).

Table (3): The rate of wound contraction per a day in the *L. europeus* oil extract group "treated and control wounds", (n=6).

Groups	Wound diameter (cm)at 0 day	Wound diameter (cm)at 11 day	Wound diameter (cm)at 14 day	Contraction rate per a day
Control(C)	2.0	0.9	0.6	0.1
Treated(T)	2.0	0.0	0.0	0.18

Table (4): Effect of *L. europeus* oil extract on macroscopic wound healing categories (Mean±SD), (n=6).

Wound duration	groups	Wound healing categories		
		Hyperemia	Exudation	Scab formation
3rd – day	Control(C)	1.66± 0.51	2.16±0.40	0.66±0.51
	Treated(T)	*2.83± 0.40	*2.83±0.40	* 1.66±0.51
7th – day	Control(C)	0.86±0.51	2.33±0.51	1.66±0.51
	Treated(T)	*0.33±0.51	*1.16±0.51	*2.50±0.54
14th - day	Control(C)	0.00±0.00	0.33±0.51	1.16±0.40
	Treated(T)	0.00±0.00	0.00±0.00	*0.83±0.57

(*) Differences between (T&C) are significant at level ($P< 0.05$).

Table (5): Effect of *L. europeus* oil extract on microscopic wound healing categories

Wound duration	Groups	Wound Healing categories				
		Neutrophils	Macrophage	Re- epithelial-zation	Granulation Tissue	
					New blood capillaries	Proliferative fibroblast +collagen
3rd - day	Control(C)	1.50 ± 0.54	0.33 ± 0.51	0.16 ±0.40	0.00 ± 0.00	0.00 ± 0.00
	Treated (T)	*2.83± 0.40	*1.83 ± 0.40	*1.66 ±0.40	0.33 ± 0.51	0.00 ± 0.00
7th - day	Control (C)	2.50 ± 0.54	0.16 ± 0.51	1.83 ±0.40	1.50 ± 0.54	1.33 ± 0.51
	Treated (T)	*1.66 ±0.51	*2.66 ± 0.51	*2.66 ±0.51	*2.83 ± 0.40	*2.16 ± 0.40
14th -day	Control (C)	0.66 ±0.51	2.33 ± 0.51	2.50 ±0.54	2.33 ± 0.51	2.33± 0.51
	Treated (T)	*0.00 ± 0.00	*3.00 ± 0.00	*3.00 ±0.00	*3.00 ± 0.00	*3.00 ± 0.00

(Mean±SD), (n=6).

(*) Differences between (T&C) are significant at level ($P< 0.05$).

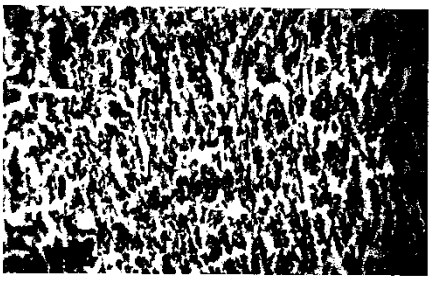

Figure 1- 3rd-day post wounding. (T) Wound, there is high infiltration of neutrophils with presence of

Figure 3- 3rd-day post wounding (C). Infiltration of neutrophils and macrophages less than in treated wounds.40X.

Figure 2- 3rd-day post wounding (T). clear re-epithiliazation40X.

Figure 4- 3rd-day post wounding (C). Re-epithiliazation less obvious than in treated wounds.40X.

Figure 5- 7th-day post wounding (T). Decrease in neutrophils infiltration, and increase macrophages. (arrow) 40X.

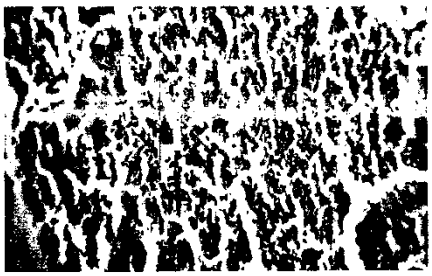

Figure 7- 7th-day post wounding (C). More infiltration of neutrophils and macrophages. 40X.

Figure 6- 7th-day post wounding (T). More obvious re-epithiliazation. 10X.

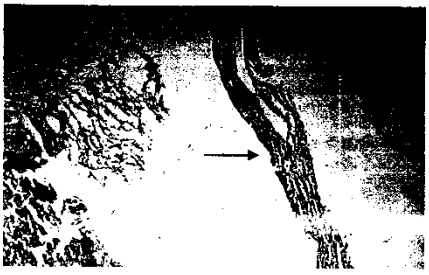

Figure 8- 7th-day post wounding (C). Re-epithiliazation less than in treated wounds. The apace between epidermis and dermis is artifactual. 40X.

Figure 9- 11th-day post wounding (T). No infiltration of inflammatory cells, complete re-epithiliazation, collagen bundles and fibroblast in between. 10X.

Figure 10- 14th-day post wounding (C). Presence of inflammatory cells, in complete re-epithiliazation.10X.

DISCUSSION

Wound contraction: In the present study, the topical application of *L europeus* oil extract on full thickness skin ulcers result in more accelerated contraction of treated wounds with 100% contraction rate at 11th-day post wounding, while in control wounds the contraction rate was 87% at the end of the experiment (14th day).

The macrophages activated by polysaccharides which stimulate the fibroblast proliferation **(23)** with subsequent proliferation of myofibroblast at the periphery of the wound, the contraction of the contractile protein of myofibroblast play important role in wound

contraction **(23-25).** Heggers *et al.* **(26)** found in their study, that the excisional wounds were treated with *Aloe vera* ointment for 14th day showed significant increase in contraction rate as compared with control wounds, the authors attributed this result, to polysaccharides in Aloe which increase collagen activity and promote wound contraction. Romo **(23),** reported a maximal rate of the wound contraction as 0.7mm/ day, depending on tissue laxity and shape of the wound, loose tissue contract more, and the square wound tend to contract more than circular wound. In the present study, the daily contraction rate of wounds treated by *L europeus* oil extract was 1.8mm/day, in spite of the tissue laxity and circular wounds, and this may reflect the efficacy of *L europeus* oil extract in promoting wound contraction.

Wound healing categories: Both macroscopic and histopathologic results, indicate that topical application of *L europeus* seeds in oil extract for full thickness cut wounds could effectively induce healing process. The presence of polysaccharides in *L europeus* oil extract, polysaccharides promote macrophage activity **(27-29).** The activated macrophages secrete cytokines such as [platelet derived growth factor (PDGF), transforming growth beta factor (TGF), Interleukins (IL), Fibroblast growth factor (FGF), Insulin growth factor (IGF-1), epidermal growth factor (EGF) and others] **(30-32).** These cytokines are essential for fibroblast proliferation, angiogenesis and chemotaxis. Among these cytokines, PDGF and Interleukins (particularly IL-1) are chemotactic and activators for neutrophils **(33-38),** and this may explain the high infiltration of neutrophils in wound treated by *L europeus* oil extract and poultice particularly in the early Inflammatory phase.

The obvious appearance of fibrovascular granulation tissue after 7th -day post wounding, may be explained by the macrophages activation with subsequent fibroblasts proliferation and their migration to wound site with collagen production **(32,36).** The macrophage- derived -angiogenic factor released in response to low oxygen tension (hypoxic condition) can explain the proliferation of new capillaries **(39-42).**

Since oxygen is required for the synthesis of collagen by fibroblast **(43),** *L europeus* oil extract and poultice may improve angiogenesis or vascular supply and make more oxygen avaible to improve collagen formation for wound healing. During the wound healing process, epithelial cells, proliferate and migrate from the edges of the wound and eventually cover the wound with newly skin **(44).** By lysing collagen enzymes, the epithelial cells move across the wound and attach to a viable tissue, the proliferation and migration of the epithelial cells is dependant on adequate supply of oxygen **(45),** therefore; the increased presence of oxygen caused by *L europeus* oil extract and poultice, improving microcirculation, should greatly

improve re-epithelization and wound healing process, this may explain the increase fibro - vascular granulation tissue and re- epitheliazation during the present experimental study.

There was a relationship between healing process and scab that covered both treated and control wounds in the two groups, but highly appearance of scab layer in treated wound of *L europeus* oil extract and less in poultice treated wound, may be due to nature of scab with can behave like semi-occlusive dressing , the role of scab has been reported by Bigbie *et al.*(46), he showed that a wound scab protect the wound, promote the migration of epithelium, and provides more cosmetically pleasing results. Apparently the scab formation in the present study bound with healing process which increased at the first period of experiment, later on later on decreased and disappeared in wounds treated with oil extract. This may due to the treated wounds in oil extract show a complete healing at 11^{th} - day of scab for treated wounds, while poultice treated wounds showed non complete healing till 14^{th}-day and this reflected by the presence of scabs in poultice and control wounds which also showed no complete healing.

Aldehydes also reported to induce healing proprieties by increased cellular proliferation and collagen synthesis at the wound site, Suguna *et al.* **(47)** found in their study on an alcoholic extract of *Centella asiatica* on rat dermal wound, the treated wound showed significant increase in the collagen content of granulation tissue, epithelialise and contract faster than control wounds, they regarded these healing proprieties due to aldehyde content in the extract of this plant, from thisone can suggest that aldehyde in *L europeus* seed may considered another growth substance induce healing proprieties, in addition to polysaccharides.

المستخلص الزيتي لبذور نبات حب الدبق يشجع التئام الجروح

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الخـــلاصـــة

اختيرت بذور نبات حب الدبق لتقييم كفاءتها في إصلاح الجروح. لهذا الغرض ،تضمنت الدراسة تحـضير الخلاصة الزيتية لبذور نبات حب الدبق. وقد أجريت تحليلات كيمياوية لهذه الخلاصة لمعرفة المحتوى الكيمياوي لها كما وتم تحضير المرهم الزيتي.

استخدمت المرهم الزيتي للخلاصة الزيتية.تضمنت هذه الدراسة (١٨) من إناث ألارانسب، قسمت بشكل عشوائي إلى ٣ مجاميع فرعية (مجموعة اليوم الثالث ومجموعة اليوم السابع ومجموعة اليوم الرابع عشر). خضع كل حيوان من كل مجموعة إلى جرحين ، جرح دائري (قطعي) متضمن كل طبقات الجلد في منطقة الظهـر. عولجـت

الجروح (المعالجة والسيطرة) بأستمرار بـ(٥ملغم) من المرهم الزيتي والفازلين على التوالي ولمدة ١٤ يوم بمعدل مرتين في اليوم.

قيمت كل الجروح في كل مجموعة تقييما عيانيا ومجهرياً. فالتقييم العياني شمل :(معدل تقلص الجرح، حساب تقلص الجرح في اليوم الواحد وقد شمل أيضا تقييم درجة احمرار الجرح، النضوح وتكوين القشور). أما مجهرياً فقد قيمت درجة (ارشاح العدلات، البلعمات، تكون النسيج الطلائي للجرح، تكاثر الاورومات الليفية مع إنتاج ألياف الكولاجين وتكون الشعيرات الدموية الجديدة).

لقد أظهرت نتائج كل من هذه المقاييس كفاءة بذور نبات حب الدبق في عمليات التام الجروح بدرجة عالية ألمعنوية (P <0. 05). اظهر كلاً من التقييم العياني للجروح والفحص النسيجي فعالية بذور نبات حب الدبق في عملية التئام الجروح بدرجة عالية المعنوية (P <0. 05). أظهرت الجروح المعالجة بالخلاصة الزيتية زيادة عالية في اليوم الثالث في كل من :درجة الاحمرار،النضوح ،تكوين القشور، ارتشاح العدلات و البلعميات والاورومات الليفية مع إنتاج الألياف الغروية وتكوين الغلاف ألطلائي الجديد ومعدل تقلص الجرح وتقلص الجرح في اليوم الواحد ، لكن حدث انخفاض في تلك العوامل في اليوم السابع ما عدا البلعمات و الاورومات الليفية مع إنتاج الألياف الغروية وتكوين الغلاف الطلائي الجديد ومعدل تقلص الجرح حيث أظهرت زيادة عالية في جروح المعالجة عند مقارنتها مع جروح السيطرة. كما أظهرت التحاليل الكيميائية الأولية للمستخلص الزيتي لحب الدبق بوجود الكلايكوسيدات والكاربوهيدرات والأديهايدات والكينونات وومجاميع التربينات والسكريات المتعددة، وعدم وجود القلويدات والفلافونويدات والسابونين.

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