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Experimental study on effect of Plantago lanculata leaves extract on contaminated excisional wound healing in rabbits

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***Abstract*---**Objective: The experimental study was performed to assessment the influence of topical application of 10% of plantago lanceolata leaves extract (PLLE) in enhancement of contaminated excisional wound in 20 adult local breed rabbits. Methodology: Twenty adult local breed male rabbits were used in this experimental study. After animals anesthetized then twenty surgical excisional wound in diameter (2cm) were created on thoracic region (1wound\side). Rabbits were divided into control group in which wounds on right side were treated by irrigation with normal saline once \daily. Whereas, in treated group wounds on left side treated by topical application of 10% PLLE ointment (once\day). All wounds were dressing. The healing process was evaluated through macroscopic examination, wound contraction rate (WCR) and histopathological examination. Result: The presence study confirm there were significantly different at (P≤0.05) were registered between control and treated group along the time of study in which PMLE play accelerating role since it used. While, the results of (WCR) indicate there is no significant difference at P≤ 0.05 at 3rd day post treatment (PT) between control group (6.75 ±0.20 A) and treated group that reach to (7.01 ±0.24 A,), but there was significant difference at P≤ 0.05 were registered in both groups of study at 7th, 14th and 21 day PT in which rapid contraction rate were recorded in PLLE group compared to control group. Histopathological results confirm that 10% of PLLE ointment cause early increased in new blood vessels formation, fibroblasts proliferation marked collagen precipitation and early reepithelization when compared with control group. Conclusion: It can be concluded that the use of 10% of PLLE

have an enhancement character of wound healing through its phenolic and flavonoid contents that act as antioxidant and anti-inflammatory substances that enhance extra fibroblast proliferation and angiogenesis.

Keywords---plantago lanculata leaves, contaminated wound healing, histopathological, grossly, rabbits, angiogenesis.

Introduction

The skin is the largest organ of the body and is the first line defense against injury (1). Skin injuries adversely affect patient survival that cause needs for healthcare wants. Wound healing processes and /or phases involve integrated cellular and biochemical cascades for reestablishment of skin's barrier strictures and function (2). The greatest common condition that is representative of a wound is bacterial infections that sometime lead to chronic inflammation (3). The authors (4) were mentioned about the role of wound management and the switch of microbial infection initiate first step of enhanced healing. In vet there are different biological or synthetics replacement were used for improve wound healing quality (5,6) as well as diode-laser also used for improve burn wounds healing time (7). Furthermore herbal medicine is important sources of novel chemical substances that have favorable therapeutic effects and have a long tradition of being used for wound treatment purposes in traditional medicine (8,9). Plantaginaceae (p.) plant is type of medical plant, it has nearly 260 species and their seeds and leaves are widely used as a medicinal benefits as a results of their various bioactive compounds, such as flavonoids, alkaloids, terpenoids, phenolic compounds, iridoid glycosides, fatty acids, polysaccharides, and vitamins (10,11). It was reported to have analgesic, anti-inflammatory, antioxidant, immunomodulatory, antifungal, anticancer effects (12). Also P. lanceolata have shown antihelminthic activity when used externally for treatment parasitic infestation in mice (13), as well as in treatment of burns, insect bites, and stomach ulcers (14,15). It was used internally to suppress coughs associated bronchitis, to reduce skin inflammation, treatment of wounds and as a laxative (16). Objective of the current study to evaluate the effect of topical application of 10% of PLLE ointment in enhancement of contaminated excisional wound healing in rabbits through grossly and histopathological examination.

Materials and Method

In-Vitro Herbs Study Methods

In the current study P. Lanceolata leaves extract (PLLE) was obtained from Swanson Health Products - USA which complies with both Food and Drug Administration (FDA) and the Federal Trade Commission (FTC) guidelines, these safeguard that gave trust for the label information and safety of these products. High performance liquid chromatography (HPLC) test was did in the Department of Environmental and Water Sciences / Ministry of Science and Technology\ Iraq for this product for detect the presence and percentage of phenols and flavonoids that consider the main ingredients in these products. Phenols and flavonoids are

works as antibacterial and free radical scavenger and they have main role in enhance infected wound healing in many researches.

How prepare of 10% of PLLE ointment

The concentration of 10% of Plantago lanceolata leaves extract (PMLE) ointment was prepared through take (0.5) g of PMLE powder and put in a sterile petri dish plate then add 9.5 g petroleum jelly and mixed well to get well distribution of PMLE particle in ointment then it storage in a refrigerator at 4^0C for used when need.

In-Vivo Study the Effect of PLLE

Experimental Animals

In the current study 40 of adult local breed male rabbits were used. They were healthy clinically, weighted 2- 2.5Kg and aged 10-14 months All animals were clinically examined and housed under the same management conditions for two weeks before starting of the experiment in animal house at the College of Veterinary Medicine/University of Baghdad. Iraq. Ethics No.248/P.G for state the study

Experimental Design

Twenty animals were used in the current study, forty excisional wound were crated on thoracic region on left and right side of animals(one wound /side), wound were divided into group A (control group) in which 20 excisional wound on right side of animals and treated by irrigation with normal saline once daily. While in group B (treated group) the other 20 excisional wounds on left side of animals, were treated by irrigation then local application of 10% of (PLLE) ointment once daily for 15 day.

Surgical wound establishment

All animals were anesthetized generally by intramuscular injection I/M of a mixture of 5mg\kg of 2% Xylazine hydrochloride and 35mg\kg of 10% ketamine hydrochloride. Thoracic region of right and left side of animal were prepared by clipping and shaving, All excisional wound size was defined by using 2cm diameter stamp to prevent any confusion in the length of wound line (fig:1-A), then sterile blade was used to created 2cm diameter full-thickness wound (1wound \side) at middle thoracic region (fig:1-B) then they were left for 24 hour for considered as contaminated wound. The 20 excisional wounds on right side were treated by irrigation with sterile normal saline (once\daily) therefore consider as control group. While in group B (treated group) the same number of wounds were treated by irrigation with sterile normal saline then 10% of (PLLE) ointment (once/ daily) applied topically for 15 day and dressing were used for both groups immediately after treatment.

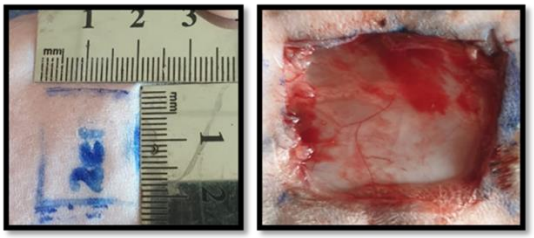

Figure.1. Photographic shows A- using of stump for detect wound size B- creation of 2cm diameter

Assessment of wound closure rate

Photographic pictures were toked for all wounds and ruler was used adjacent to the wound border at 3rd, 7th, 14th and 21 days post initial wound treatment. The images were processed by using amorphometric software (Image J®, National Institute of Health, Bethesda, MD, USA to assess the wounded areas during each period for both groups. Then wound closure rates were obtained according percentage of the reduction in original wound area size by using the following formula (17,18).

Wound contraction (%) = (Ao-At) / Ao ×100

Where, A0 is the initial wound distance while At represents the distance of the wound at the time of photo capturing (3rd, 7th, 14th and 21 day respectively).

Histopathological assessment of the wound healing

The specimens of wound site were taken for all animals at 3rd , 7th , 14th and 21 day after treatment, then they irrigate with tap water for removal of any blood or clot and fixed immediately in 10% buffered formalin solution then set in paraffin. The wound tissue contained dermis and epidermis, they sectioned to prepare 5-7 µm thick and stained with hematoxylin-eosin (H&E) stain and special stain Mallory Trichrome stain. Histopathological slides were prepared and the whole procedure was performed by the Department of Pathology, Collage of Veterinary Medicine / University of Baghdad/ Baghdad. Iraq.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

Results

Results of HPLC test for (PLLE)

In the current study the result HPLC analysis of PLLE sample proved that they have phenolic compounds like (Pyrogallol, gallic acid, caffeic acid, cinnamic acid,

catechol, 4-hydroxyl benzoic, cinnamaldehyde, eugenol, lignin, chlorogenic, nigellone) that have antiseptic and antioxidant function. In addition to flavonoid compound also extant such as (rutin, kaempferol, vanillic acid, and qurctin) were proved in PLLE that have antioxidant and anti-inflammatory function.

Macroscopical Examination

The results of macroscopical wound healing and contraction was shown in Fig (2) in which at 3rd day PT showed the scab was covered wounds surface with start of irregular wound contraction in both group and maintain the same size grossly. While at 7th day PT showed different in progression of wound healing by decrease in size of wound scab and marked start peripheral reepithelization character by pinkish colour of border in PLLE group with well granulation tissue formation, on other hand slowly wound healing and contraction was showed in control group with still scab persistence. At 14th day PT showed more advance in reduction of wound size semi complete wound closure and reepithilization and spot like wound appear with sparse hair growth at periphery but less advance while wounds of control group that showed disappear of scab with well granulation tissue formation in addition to progress of wound contraction and reepithelization. At 21 day PT the macroscopical examination was concluded that the fastest re-epithelisation was observed in wounds treated by PLLE. In control group there were shown quiet small spot of wound not closed and scab still presence with sparse hair growth at periphery while scar tissue was replaced wounds of both treatment group but in less area in PLLE group with increased in distribution sites of hair growth.

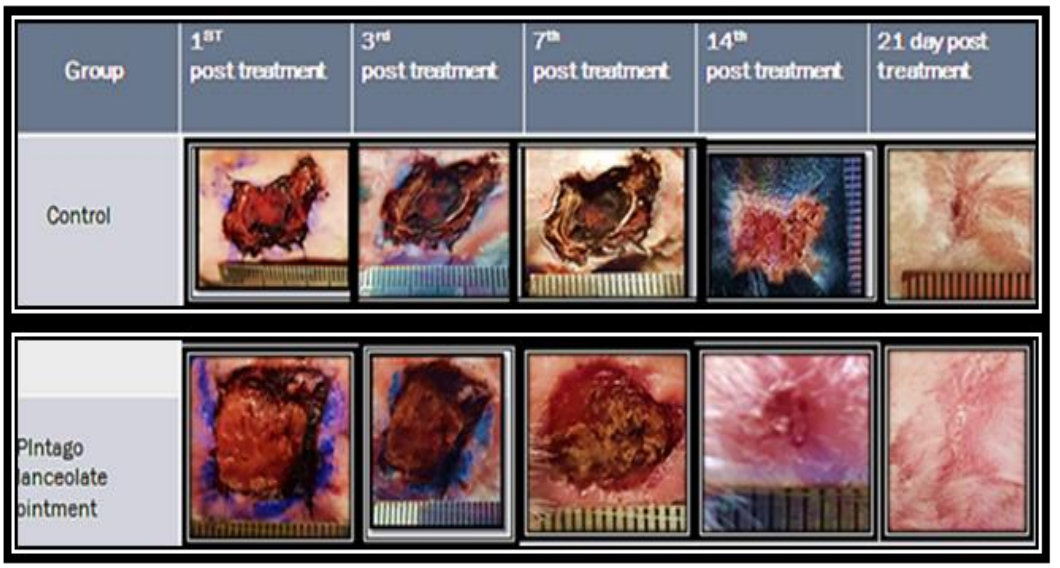

Figure 2; Photographic shows the wound healing macroscopically in control group and PLLE groups at 1st 3rd,7th, 14th ,and 21 day post treatment

Results of wound contraction rate

The results of wound contraction rate of the current study for both groups show in table (1). This results indicate incidence of significant difference at P≤ 0.05 along the periods of study in control group that showed increased in wound contraction rate with time of study as recorded (6.75 ±0.20 d) at 3rd day PT, and progress to (17.95 ±0.20 c) then (68.54 ±0.80 b) and reach (90.25 ±0.76 a) at 21 day PT. While in the PLLE group showed significant difference at P≤ 0.05 along time of study that recorded (6.82 ±0.13 d) at 3rd day PT (23.01 ±0.40 c) at 7th day PT, then (84.24 ±0.54b) at 14th day PT and (100.00 ±0.00 a) at 21 day PT.

In the current study there is no significant difference at P≤ 0.05 of wound shrinking percentage at 3rd day PT between control group (6.75 ±0.20 A) and treatment groups (6.82 ±0.13 A). But there was significant difference at P≤ 0.05 were registered in percentage of shrinking wounds in all groups of study at 7th, 14th and 21 day PT, at 7th day the control group shows less percentage of shrinking (17.95 ±0.20 C) while well contraction rate registered in PLLE group that reach (23.01 ±0.40 B). At the 14th day PT recorded significant difference at P≤ 0.05 was recorded (68.54 ±0.80C) in control group but more increased in PLLE group (87.24 ±0.54 B). as well as at 21 days PT there were registered significant difference at P≤ 0.05 in percentage of shrinking wounds in control group (90.25 ±0.76 B) and complete closured was registered in PLLE group (100.00 ±0.00 A) at this time.

Table 1: Shows the Means ± SE of the wound contraction rate %for control and PLLE group at 3rd,7th ,14th ,and 21 day post treatment

Group	Mean ± SE of Wound contraction				LSD value
	Day 3	Day 7	Day 14	Day 21	
Control	6.75 ±0.20 A d	17.95 ±0.20 C c	68.54 ±0.80 C b	90.25 ±0.76 B a	8.63 *
Plantago Lanceolata	6.82 ±0.13 A d	23.01 ±0.40 B c	84.24 ±0.54 B b	100.00 ±0.00 A a	7.05 *
LSD value	0.565 NS	0.981 *	1.870 *	1.244 *	---
Means with different big letters in the same column and small letters in the same row are significantly different. * (P≤0.05).					

Histopathological Results

Group A (control group)

The results of histopathological sections at 3rd day post wound revealed presence of very thick fibrin clot which covered the injury site that cover the thick layer of necrotic tissue formation that was separated from underlying dermis by zone of dead polymorphonuclear cells (PMNCs) in addition to little fibrin framework formed as in (fig.3.A). While at 7th the wound area was still covered by remnant of the clot with initiate formation of thin line of epithelization at the border of wound. The area of dermis characterized by formation of granulation tissue and

immature collagen fibrils that associated to fibroblast proliferation, the sections revealed poor angiogenesis (fig. 3.B and C).

The histopathological sections at 14th day revealed well epithelization at wound surface which characterized by thin layer of non- keratinized stratified squamous epithelium that showed mitotic (fig.3.D). The area of dermis layer was composed of still organized granulation tissue that contain numerous of fibroblasts and immature collagen fibrils, the dermis revealed no sebaceous glands and no hair follicles (fig.3.E). The histopathological sections of wound site at 21 day revealed that the thickness of epidermis was improvement than prior period associated with formation of thick layer of keratinized stratified squamous epithelium, as well as the dermis was composed of mature granulation tissue that formed dermal papilla, contained numerous of fibroblasts and mature collagen bundles, the dermis revealed no sebaceous glands and no hair follicles (fig. 3.F).

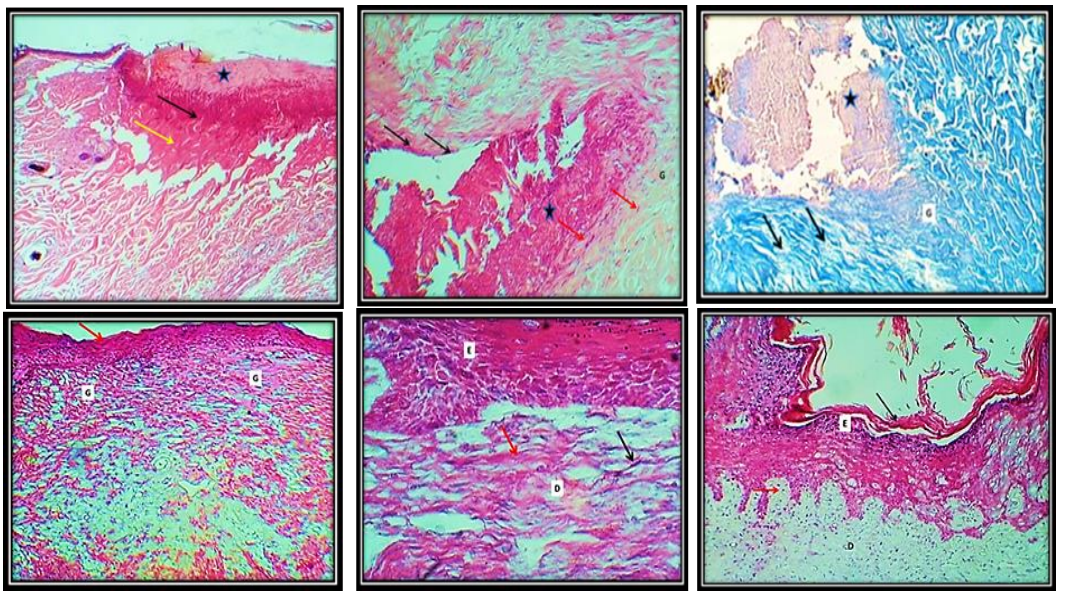

Figure 3: Histopathological sections of wound site in (GA) A- At 3rd day P.T shows fibrin clot (Asterisk), necrotic tissue (Black arrow) and little fibrin deposit (yellow arrow). H&E 10X. B- At 7th day P.T shows: remnant of necrotic tissue (Asterisk) & thin line of epitheliazation (Black arrows) & fibroblasts proliferation (Red arrows) & granulation tissue formation (G). H&E stain. 10X. C- At 7th day P.T shows still remnant of clot (asterisk), while the dermis area notice formation of granulation tissue with immature collagen fibrils (Arrows) Masson stain.10X. D- At 14th day P.T shows thin layer of epidermis (Red arrows), while the dermis composed of organized granulation tissue (G). H&E stain. 10X. E- At 14th day P.T shows stratified squamous epithelium (E) within immature collagen fibrils (Red arrow) and fibroblast (Black arrow) H&E stain. 40X. F- At 21 day P.T shows: thick layer of stratified squamous epithelium (E) with thick keratin layer (Black arrow), & dermis (D) with mature granulation tissue formed dermal papilla (Red arrow). H&E stain. 10X.

Group C (PLLE) ointment

The histopathological sections of the wound site at 3rd day PT indicated the formation of very thick layer of fibrin clot followed by thick zone of necrotic tissue and infiltration of inflammatory cells mainly (PMNCs). Little aggregation of fibroblast cells and edema were presence in dermis layer. The signs of regeneration was mild at the deepest part of dermis that characterized by little proliferation of fibroblast cells (fig. 4-A). The results of histopathological examination at 7th day PT revealed presence of thin layer of fibrin clot followed by remnant of necrotic tissue. The dermis layer manifested by well angiogenesis formation that characterized by many of newly formed blood vessels, and so on the granulation was well organized with furthermore of fibroblasts in addition to formation of mature and immature collagen bundles. (fig. 4 B and C).

The histopathological sections of wound site at 14th day revealed complete epithelization of epidermis that composed of slightly thick layer of keratinized stratified squamous epithelium. The dermis layer also revealed immature collagen fibrils and numerous buds of hair follicles (fig. D and E). While the sections of wound site at 21 day revealed the epidermis was composed of slightly thick non keratinized stratified squamous epithelium. The dermis revealed presence of mature collagen bundles that formed numerous dermal papillae and enriched with hair follicles (fig. F).

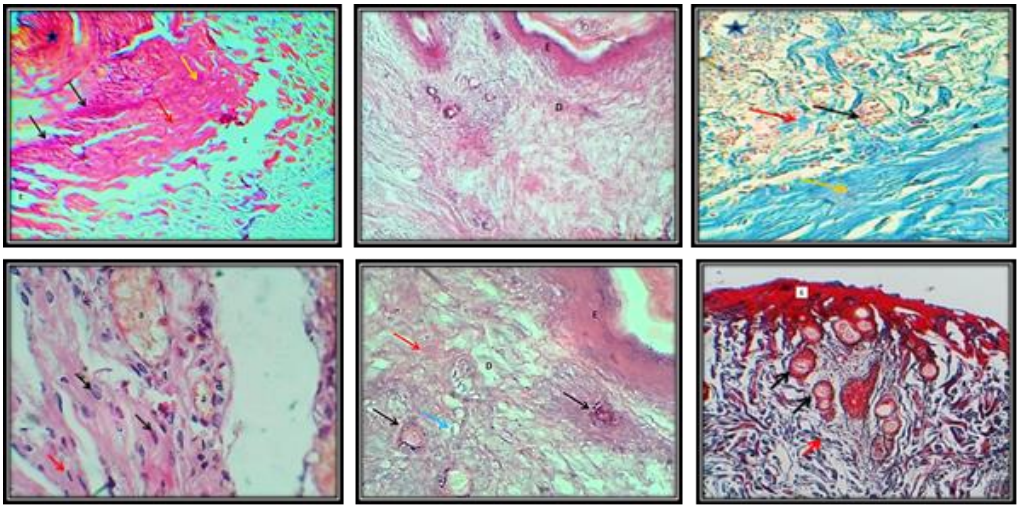

Figure 4- Histopathological sections of wound site (GB) A- At 3rd day P.T with (PLLE) shows Slightly thick fibrin clot (Asterisk), necrotic tissue (Black arrows), thick layer of fibrin deposit (Red arrows), little of fibroblasts proliferation (Yellow arrow) and edema (E). H&E stain, 10X. B- At 7th day P.T with (PLLE) shows presence of necrotic tissue (Asterisk) and fibroblasts with immature collagen fibrils (Arrows) and well angiogenesis (a). H&E stain. 40X. C- At 7th day P.T with (PLLE) shows thick immature collagen fibrils (Red arrow) and numerous BVs formation (Black arrow) Masson stain 40X. D- At 14th day P.T with (PLLE) shows slightly thick layer of epidermis (E) & dermis (D) with mature granulation tissue. H&E stain. 10X. E- At 14th day P.T shows keratinized stratified squamous epithelium (E), the dermis (D) with immature collagen fibrils (Red arrow),

fibroblast (Blue arrow) & buds of hair follicle (Black arrows). H&E stain 40X. F- At 21 day PT with (PLLE) shows epidermis (E) & dermis (D) rich with collagen bundles (Red arrow) and hair follicles (Black arrows). Masson trichrom stain. 10x.

Discussion

When the skin is injured, there is the possibility of local bacterial infection, resulting in the delay of healing process (19).In response to infection, the body starts an inflammatory mechanism includes migration of leukocytes and cytokine release (20). However, the phagocytic activity of leukocytes leads to the release of endotoxins by the bacteria, resulting in local necrosis and inflammation due to the increase of pro-inflammatory cytokines, higher metalloproteinase activity and decrease of growth factors release (21). Although an immediate inflammatory response is an initial physiologic mechanism of healing, while chronic inflammation impairs the healing process, affecting re-epithelialization and delaying wound retraction and tissue remodeling (20,21).

The result of HPLC for PLLE sample proved its have the phenolic compounds and accepted to the results of (22), who were found the same a content of phenolic compounds when they examined PLLE by HPLC analysis. As well as Phenols have been reported to be the main bioactive compounds responsible for antioxidant activities of P. lanceolata by (23,24).

The macroscopical result of the current study give an indication to the potential effect of PLLE that accelerate contaminated wound healing when compared with control group as showed at 7th day and still along 14 and 21 day PT. This result may be related to two main factors first one the contents of PLLE recorded by HPLC analysis such as phenolic compounds that have antiseptic and antioxidant function, in addition to flavonoid compound that have antioxidant and anti-inflammatory function. These results agree with the authors (4) who mentioned about the role of wound management and the control of microbial infection lead to better healing.

In the current study PLLE have a stimulation role in accelerate wound healing may be through inducing stimulation of fibroblast proliferation as showed that PLLE have an important effect in accelerate wound healing when compared to control group that used only irrigation with normal saline and the macroscopic results of the came in line with the results of a study by (25) whom reported Plantago extract spp. has other effects, such as increased nitric oxide production (NO), which may play a role in the enhance wound healing. All these results agreement with a study by (26) who related their results to astringency effect of flavonoids which may play a key role in accelerating wound healing.

The current study confirmed that moist dressing or topical PLLE ointment application in group B have a well effect in wound healing as compared to control group. This result also come to an agreement with a study by (27) who referred moist wound environment cause shorten and accelerate wound healing process through enhance epidermal migration, promote angiogenesis and connective tissue synthesis. As well as other authors (28,29) whom reported that dry wound

tends to heal slowly when compared with wound treated in a moist environment due to impaired epithelialization and scab formation.

The positive results for wound healing acceleration in treated group give advantage for dressing that used in the current study also explained that the dressings help in faster epithelialization, collagen synthesis, promote angiogenesis by creating hypoxia to the wound bed and decrease wound bed pH which leads to a decrease in the wound infection as mention by (30), in addition P. spp. have a role in reduction of inflammation it when used as a moist dressing. And in other studies by (31, 32) who explained shortens the inflammatory healing phase associated using of moist dressing and produces good cosmetic results.

The results of wound contraction rate in the current study showed at 7th day in both groups were came in line with results of many studies by (18, 33) whom mentioned to the events of wound contraction begins approximately 4 to 5 days after initial injury due to first appearance of Myofibroblasts and it is a cell-directed process: cell division is required, but collagen synthesis is not. In a non-sutured wound, wound edges move toward one another at a rate of approximately 0.6 to 0.75 mm/d. This process correlates in time with phenotypic morphogenesis of fibroblasts into myofibroblasts.

The histopathological result of the current study gave an indication about the potential effect (PLLE) in accelerate wound contraction when compared with control group that registered at 7th day and still along 14 and 21 day PT. These results may be related to the ratio of its contents and the effective percentage of PLLE on healing process of wounds. The same results of healing acceleration confirmed by (34,35,36) whom indicated Plantago species effect on the proliferation of fibroblasts resulting in the wound healing acceleration in their studies and this effect related to polyphenolic compounds in addition to antioxidant and antibacterial substance in plantago species may be responsible for healing properties of extract as well as the proteins extracted from.

Furthermore, the histopathological results of the current study have acceptance with results by (37) when induced an excisional 1 cm wound in the skin on the back of the mice in all groups. An ointment containing 10% PLLE was applied to the wound in group 1, an ointment containing 20% PLE was applied in group 2 and vaseline was applied in group 3. Results of their study at 14 day confirmed that epithelialization was more prominent in group 2, while vascularization and collagen deposition was more advanced in groups 1 and 2 compared to the other groups. These results disagree with the results of study by authors like (15) that did not showed any significant differences on animal rat model between control and PLLE treated groups at dose of 5 and 10%. In conclusion the current study confirm that PLLE have a stimulation role in accelerate contaminated wound healing through inducing stimulation of fibroblast proliferation, and angiogenesis and PLLE has an important effect in wound healing when compared to control group that used only irrigation with normal saline.

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