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Experimental study of the effect of *Plantago major* leaves extract on contaminated excisional wound healing in rabbits

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Abstract

The current study was performed to estimate the influence of topical application of 10% of *Plantago major* leaves extract (PMLE) on a healing process of contaminated excisional wound in local breed rabbits. Twenty adult local breed male rabbits were used. After the animals have generally anesthetized, two 2×2 cm full thickness wounds were created in thoracic region on right and left side (1wound/side) for each animal. Wounds on right side of animal were treated by topical irrigation with normal saline/daily, this consider as control (group A). While, left side wounds were treated by topical application of 10% PMLE ointment (once/day) that consider as treated (group B) dressing was used after each treatment for both groups. Wound healing was evaluated through macroscopic examination, wound contraction rate (WCR) assessment and histopathological examination. Results of macroscopic examination confirmed that PMLE has a role in acceleration healing when compared to control group. These outcomes were parallel with WCR results in which reflect the mean rate of wound contraction on days 7^{th}, 14^{th}, and 21^{st} in PMLE-treated group was significantly higher ($P<0.001$) than that of the normal saline-treated group. Histopathological examination results confirmed early increased in new blood vessels formation, fibroblasts proliferation, marked collagen precipitation and early epithelization in group PMLE compared to group A. This study confirmed that topical application of 10% of PMLE (once/a day) has an effective role in accelerate contaminated wound healing through its phenolic and flavonoid contents as recorded by high performance liquid chromatography (HPLC) assessment that act as antioxidant and anti-inflammatory substance which help in the enhancement the WCR through encourage early and additional fibroblast proliferation and angiogenesis when compared to control group.

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Introduction

The largest organ of the body and the first line of defense against injury is a skin, which plays critical roles in maintaining homeostasis (1). Wound lead to an opening or breaking of the skin. The incidence of bacterial invading of wound permitting to cause inflammatory response is too excessive and the repair process become prolonged, however sub infective levels of bacteria appear to accelerate wound healing and formation of granulation tissue (2,3). Muhammad and Muhammad (4) mentioned about the role of wound management and the control of microbial infection led to encourage healing process (4). There are several experimental studies were achieved for enhance excisional wound healing through using different materials such as biological or synthetics replacement and showed have some positive effects on accelerate wound healing (5,6), or by using diode-laser for burn wounds in rabbits (7). In addition, many plants species contain bioactive compounds and have a long tradition of being used for wound treatment purposes

in traditional medicine (8). A review on medical drugs in leader countries was reported 25% of the medical drugs were discovered based on plants and their derivatives specially in India and China and used widely in for treatments (9,10) due to theirs prophylactic or therapeutic efficiency, low toxicity, and side effects as compared with the synthetic drugs (11). Plantaginaceae (p.) plant is type of medical plant that widely used, it has about 260 species; they were found in temperate regions and in tropical zones; and the most famous species are *P. major*, *P. ovate*, *P. media*, *P. lanceolata*, *P. indica*, and *P. asiatica* they have been developed as medicines for thousands of years due to they possess considerable bioactivity and contain beneficial phytochemicals (12-15). Medicinal benefits of the seeds and leaves of Plantaginaceae plant may be related to various bioactive compounds, such as flavonoids, alkaloids, terpenoids, phenolic compounds, iridoid glycosides, fatty acids, polysaccharides, and vitamins (16,17). There were reported to have analgesic, anti-inflammatory, antioxidant, immunomodulatory, antifungal, anticancer effects (16). The therapeutic effect f of *P. major* in treatments of acute urticarial in patient was studied (18), While Ghanadian *et al.* (19) describe the well efficiency role of using 10% *P. major* leaves extract in treatment of diabetic foot ulcer (DFU) and pressure ulcer (19). In addition, it was used in Malaysia country as therapeutic drug for diuretic, tonic, and coughing (20) and to treat urinary calculus (21) and diabetes (22).

Objective of the current study was to evaluate the effect of topical application of *P. major* leave extract in a concentration of 10% in enhancement healing of contaminated wound in rabbits through macroscopic and histopathological examination.

Materials and methods

Ethical approval

The experimental design and procedures used in this study were reviewed and approved in accordance with animal welfare ethical standards by the Department of Veterinary Surgery and Obstetrics, College of Veterinary Medicine, University of Baghdad, in its session held on October 5, 2020, and the Scientific Committee at the University of Baghdad's College of Veterinary Medicine with ethics No. 248/P.G.

In-Vitro herbs study methods

In the current study *P. major* leaves extract (PMLE) was obtained from Swanson Health Products - USA that prepared leaves extract through 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 done 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 studies.

Plant percentage preparation

The concentration of 10% of PMLE ointment was prepared according to Ghanaian *et al.* (19) then stored at 4°C for use as needed.

Experimental Animals

In the current study 20 adult local breed male rabbits were used. They were healthy clinically, weighted 2- 2.5 kg and aged 10-14 months. All animals were clinically examined and housed under the same management conditions in animal house at the College of Veterinary Medicine, University of Baghdad, and acclimatized for two weeks before starting of the experiment.

Experimental design

Twenty animals were used in the current study and all which consider as group A and B according to the wound site that created in the left or right side of animal and the materials were used for topical wound treatment. Group A (control group) the 20 excisional wounds (one wound /side) in thoracic region on right side of animals were treated by irrigation with normal saline once daily. While in group B (treated group) the 20 excisional wounds (one wound/side) on thoracic region on left side of animals, were treated by irrigation then topical application of 10% of (PMLE) ointment once daily for 15 days.

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 (23). Wound site was prepared by clipping and shaving, all excisional wound size was defined by using square stamp (2×2cm) to prevent any confusion in the length of wound line (Figure 1-A), then sterile blade was used to created full-thickness wound (1wound /side) in middle of thoracic region (Figure 1-B), they were left for 24 hours for considered as a contaminated wound.

In group A the 20 excisional wounds on right side were treated by irrigation with sterile normal saline (once/daily). While in group B (treated group) the 20 excisional wounds were treated by irrigation with sterile normal saline then 10% of (PMLE) ointment (once/ daily) (Figure 2-A), this step of treatment was repetitive for 15 days. Dressing was used for animals of each group immediately after treatment (Figure 2-B).

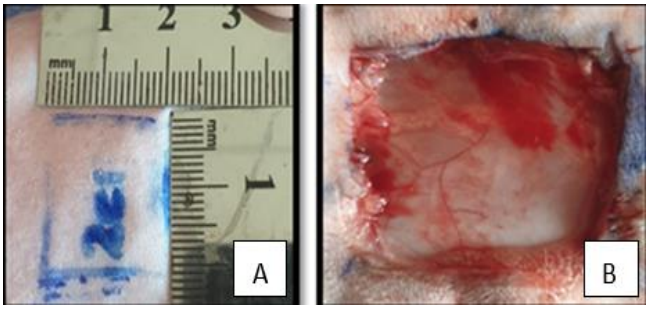

Figure 1: Photographic shows A- using of stump for detected wound size B- creation of 2cm diameter.

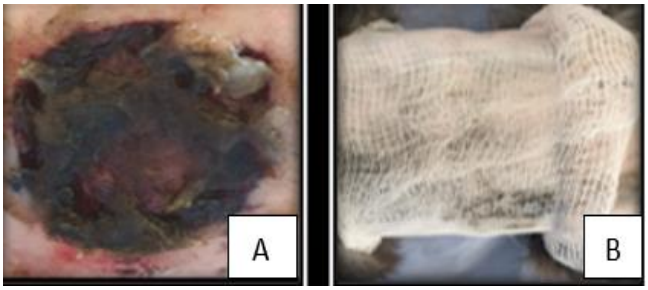

Figure 2: Photographic shows A- wound site in group B covered by 10% PMLE ointment. B- Dressing of wounds thoracic region in left and right side of rabbit.

Assessment of wound closure rate

This study was continued for 21day after the creation of surgical wound. Wounds were photographed at 3rd, 7th, 14th and 21 days PT. Ruler was used adjacent to the wounds were photographed and wounds were considered to have healed when visible epithelium covered the wound and cicatrisation and pigmentation was found. The images were processed 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 (24,25).

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

Where, Ao is the initial wound distance while at represents the distance of the wound at the time of photo capturing (on day 0, 3rd, 7th, 14th and 21 respectively).

Histopathological assessment of the wound healing

The specimens of wound site were taken for all animals at 3rd, 7th, 14th and 21st days after treatment, then there were fixed, sectioned and stained with haematoxylin-eosin (H&E) stain (26) and special stain Mallory Trichrome stain (27). 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

Data on wound contraction rate were subjected to two-way analysis of variance (ANOVA) using the Standard Least Squares procedure of JMP Pro 14.0 software (SAS, Institute Inc., Cary, NC, USA). As fixed factors, the model included treatment (control, PMLE), time (days), and their interaction (treatment × time). Boxplot analysis was performed to determine the presence of potential outliers, and they were eliminated to ensure that data were normally distributed. Using the Tukey's honest significant difference (HSD) test, significant main effects and interaction means were separated and accepted at $P \leq 0.05$.

Results

Results of HPLC test for PMLE

In the current study the result HPLC analysis of *P. majoir* (Leaf) 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, flavonoid compound also extant such as (rutin, kaempferol, vanillic acid, and qurctin) were proved in *P. major* that have antioxidant and anti-inflammatory function.

Results of wound contraction rate

The results of wound contraction rate of the current study for both groups are presented in table1. Except for day 3 of wound treatment, a significant treatment × time interaction effect ($P<0.001$) was observed constantly throughout the study period for wound contraction rate response, where the mean rate of wound contraction on days 7th, 14th, and 21st in PMLE-treated group was significantly higher ($P<0.001$) than that of the normal saline-treated group. Among the periods of study, in control group showed gradual increased in wound contraction rate with time it was recorded 6.75±0.20 on 3rd day then 17.95±0.20 on 7th day while recorded (8.5±0.80 on 14th day and reach 90.25±0.76 on 21st day post treatment (PT). While in PMLE group it was noted 7.01±0.24 on 3rd day PT then progress to 25.10±0.39 on 7th day then recorded 92.88±0.61 on 14th day, while on 21st day it was registered 100±0.00 that consider more advance and completely closured when compared to control group at this time (Figure 3).

Table 1: Effect of *Plantago major* leaves extract (PMLE) as topically applied at 10% on wound contraction rate of contaminated excisional wound

Time (days)	Control group	Major group	Main Effect Time	P-value (LSD)
3	6.75±0.20 ^{g}	7.01±0.24 ^{g}	6.88±0.16 ^{W}	<0.001 (4.18)
7	17.9±0.20 ^{f}	25.1±0.39 ^{e}	21.5±0.61 ^{Z}	
14	58.5±0.80 ^{d}	92.9±0.61 ^{b}	75.7±2.79 ^{Y}	
21	90.3±0.76 ^{c}	100±0.00 ^{a}	95.1±0.87 ^{X}	
Main Effect Treatment	43.4±3.75 ^{B}	56.2±4.59 ^{A}		
P-value (LSD)	<0.001 (11.7)			
Treatment × Time				
P-value (LSD)	<0.001 (2.08)			

Values are means±SEM, n = 20 per treatment group. $^{A-B}$ Means followed by different uppercase letters in the same column are different from each other in Treatment factor. $^{X-W}$ Means followed by different uppercase letters in the same column are different from each other in Time factor. $^{a-g}$ Means followed by different lowercase letters in the same column or row are different from each other in interaction effect Treatment × time.

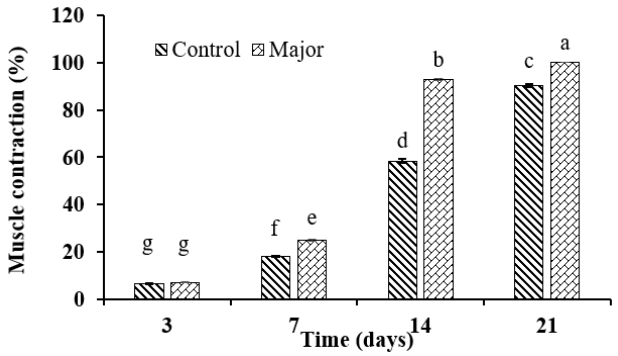

Figure 3: Diagram evaluate the effect of *Plantago major* leaves extract (PMLE) as topically applied at 10% on wound contraction rate (%) of contaminated excisional wound in local Iraqi breed rabbits.

Results of macroscopical examination

The results of macroscopical examination of wound healing were shown in figure 4 in which at 3rd day PT the scab was covered wounds surface with start of irregular wound contraction in all 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 with marked start peripheral epithelization character by pinkish color of border especially in PMLE group, in the other hand slowly wound healing and contraction was showed in control group with still scab persistence. At 14th day PT showed more advance in wound healing especially in PMLE group that showed semi complete wound closure and re-epithilization and spot like wound appear with sparse hair growth at periphery,

At 21-day PT the macroscopical examination can be concluded that the fastest reepithelisation was observed in wounds treated by PMLE. 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 PMLE group with increased in distribution sites of hair growth.

Histopathological group A (control group)

The results of histopathological sections at 3rd day PT 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 (Figure 5-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 (Figure 5-B and 5- 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 (Figure 5-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 (Figure 5-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 (Figure 5-F).

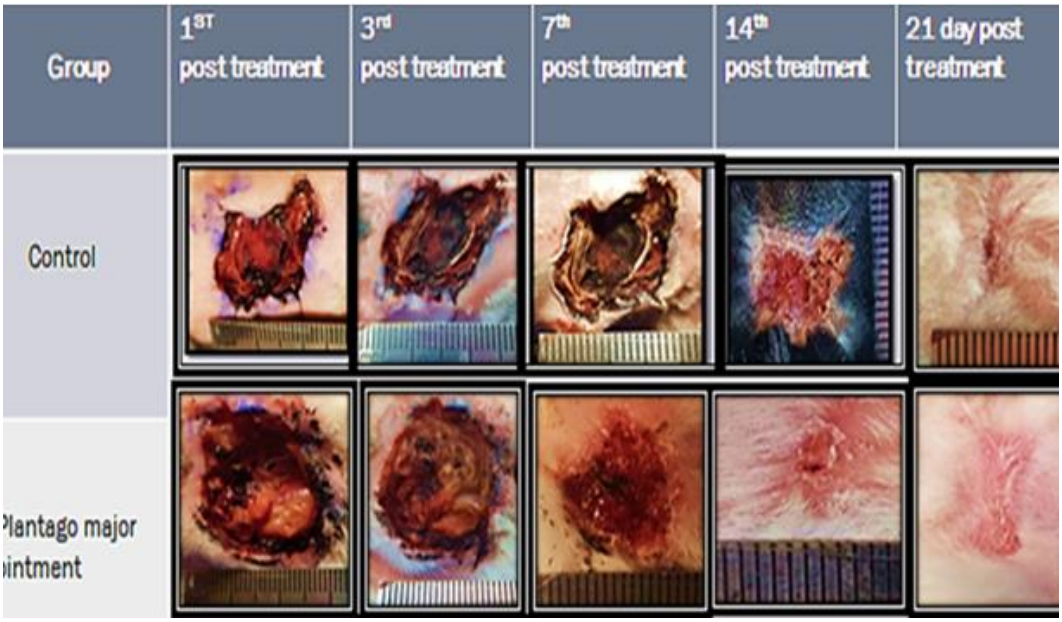

Figure 4: Photographic image shows advancement of healing in group A and B at 1st, 3rd, 7th, 14th and 21 day post treatment.

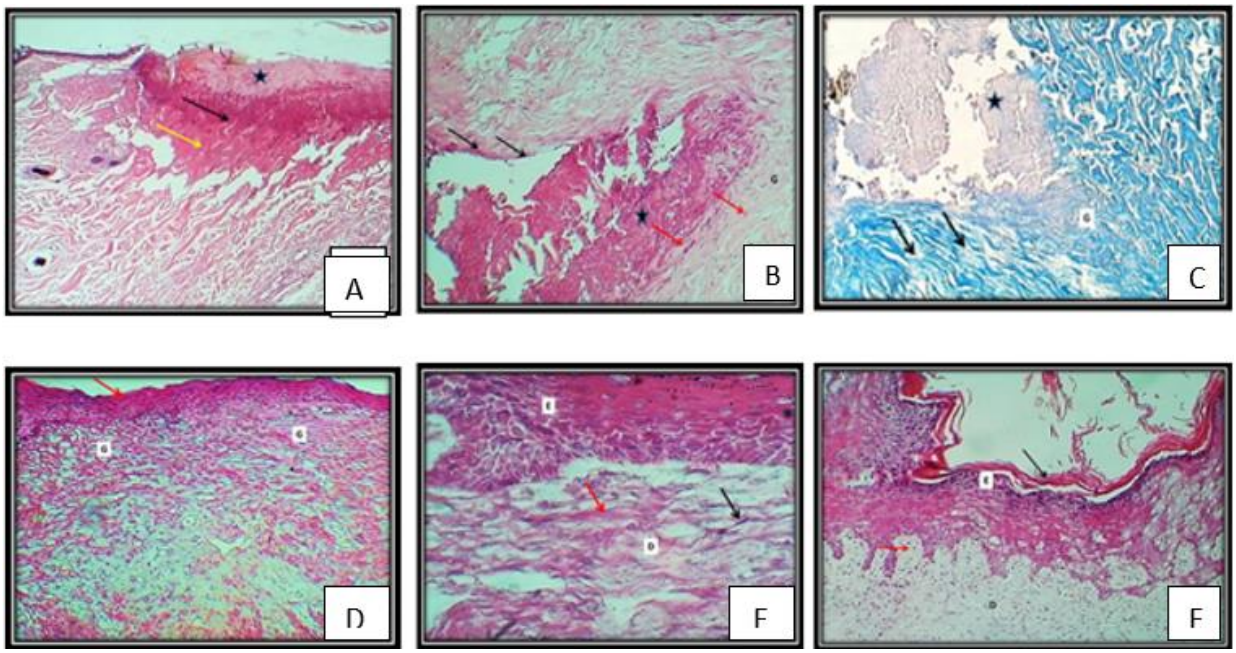

Figure 5: Histopathological view of wound site of group A A- At 3rd day P.T shows fibrin clot (Asterisk), necrotic tissue (Black arrow) and little fibrin deposit (yellow arrow). H&E 10X. B- At 7th day P.T shows remnant of necrotic tissue (Asterisk) & thin line of epithelization (Black arrows) & fibroblasts 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.

Histopathological group B (PMLE) ointment

The histopathological sections of skin in wound area revealed thick layer of fibrin clot which followed by a thin zone of necrotic tissue with little inflammatory cells infiltrates, in addition in other section showed sever deposition of fibrin, with marked proliferation of fibroblast and edema, while other section which revealed marked immature collagen fibrils in addition to several blood capillary formations (Figure 6-A). Histopathological sections of the wound dermis site at 7th showed very well angiogenesis that characterized by furthermore of newly formed blood vessels distribution, and large area of granulation was well organized with furthermore of fibroblasts and immature collagen bundles (Figure 6-B). At this time marked of epithelization was showed at the wound border (Figure 6-C). While at the 14th day the histopathological sections of wound site exposed complete epithelization of epidermis that composed of thick layer of keratinized stratified squamous epithelium more than showed in control group contained hair follicle (Figure 6-D). The dermis also revealed well organized granulation tissue with mature collagen bundle and fibroblast (Figure 6-E). The last period (21 day) recorded through the histopathological sections of wound site that the epidermis was composed of very thick layer of non-keratinized stratified squamous epithelium with mature collagen bundle. Other section of epidermis and dermis revealed presence of mature collagen bundle, fibroblasts and fibrocytes, hair follicle and blood vessel, there were no inflammatory cells (Figure 6-F).

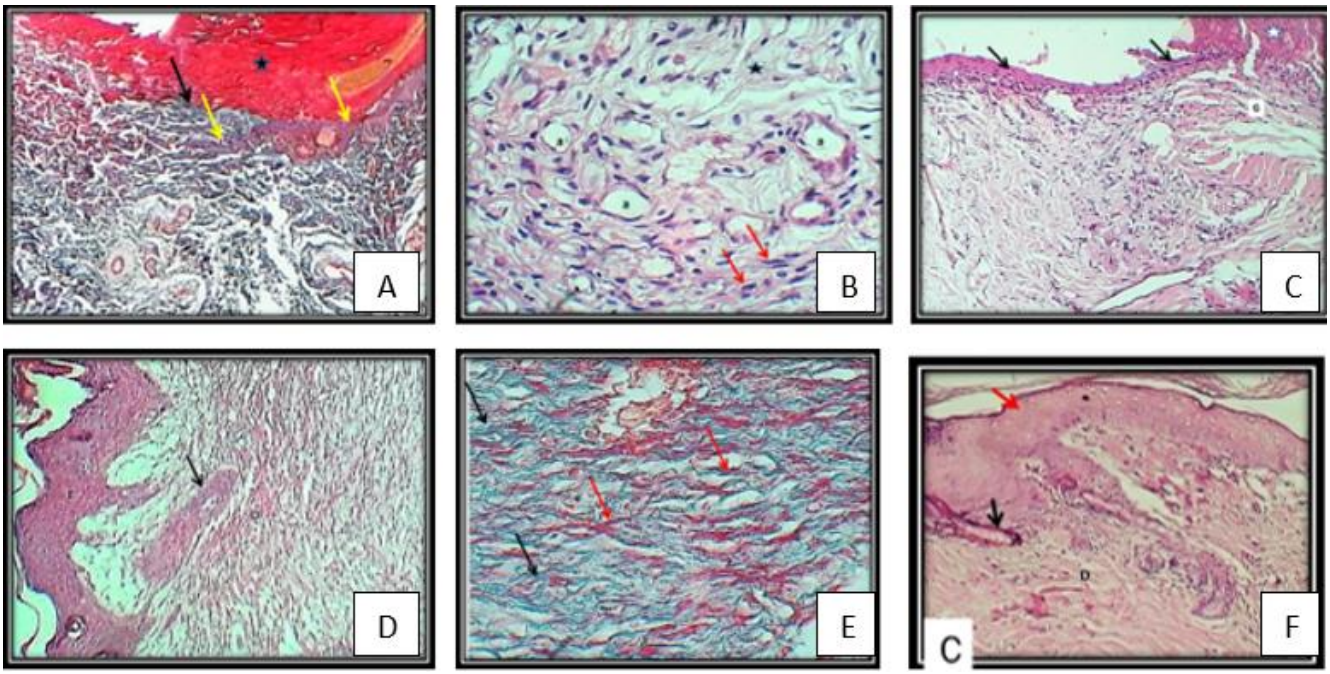

Figure 6: A- Histopathological view of wound site of group B A- At 3rd day P.T shows necrotic tissue formation (Asterisk) & framework of fibrin deposits (yellow arrows) with inflammatory zone formation (Black arrow) and several new blood capillary formations. Masson's trichrome stain 10X. B- At 7th day P.T shows progress in proliferation of fibroblasts (Red arrows) within immature collagen fibrils (Asterisk) and well BVs distribution (a). H&E stain. 40x. C- At 7th day shows presence line of epithelization at rim of injury (Arrow) wide is of granulation tissue (G) and necrotic tissue still shows (asterisk). H&E stain. 10X. D-At 14th day P.T shows thick layer of epidermis (E) & dermis (D) with mature granulation tissue contained hair follicle (arrow). H&E stain. 10X. E- At 14th day shows well organized granulation tissue of dermis with mature collagen bundle (Black arrows) & fibroblasts (Red arrows) Masson's trichrome stain. 40X. F-At 21 day P.T shows very thick epidermis (Red arrow) and granulation tissue of dermis with mature collagen bundles (D) and hair follicles (Black arrow).H&Es stain. 10X. D- At 21 day shows epidermis€ and dermis with mature collagen bundles (D), blood vessels (red arrows) and keratin layer (Black arrow). H&E stain.10×.

Discussion

Skin injuries adversely affect patient survival that cause needs for healthcare wants (28). The *Plantago major* plant has a long history in traditional medicine in the treatment of wound and several diseases. From the perspective of modern medicine, this plant contains a number of safe compounds like flavonoids, alkaloids, terpenoids, phenolic compounds, iridoid glycosides, fatty acids, polysaccharides, and vitamins (13,14).

In this study, the animals of treated group with PMLE showed a high percentage of (WCR) started from the 7th day P.T advanced to the end of study compared to control group. These results indicate that topical application of PMLE once a day in a percentage 10% was the best concentration for the treatment of contaminated wound.

This study has evaluated the effect of 10% of *P. major* on contaminated wound. This agreed with Mahmood and Phipps, (29) who used of 5 and 10% PMLE ointment and confirmed its role in accelerated the wound healing in rats compared to the control group (Vehicle alone) (29). Furthermore, these results were consistence with results of Ghanadian *et al.* (19) when used of 10% topical gel of PMLE in diabetic foot ulcer (19). As well as Kartini *et al.* (30) who confirmed that the topical application of PMLE on rats' wounds play a role in increased wound closure and fibroblast proliferation significantly at ($P<0.05$), particularly on 7th and 14th day (30).

This wound healing enhancement may be related to two main factors. First, the contents of PMLE the HPLC analysis in this study corroborate that PMLE contain phenolic compounds which has antiseptic and antioxidant function, in addition to flavonoid compound that have antioxidant and anti-inflammatory function. Second, due to the PMLE high percentage 10% used which has proven to be the causes of the enhancement of healing process of wounds. In the other hand, the PMLE have an effect on reduction of bacterial growth by inducing stimulation of fibroblast proliferation.

These suggestions were compatible with other researcher who confirms that polyphenolic compounds and antioxidant properties of the extract are the major reasons of wound healing by using PMLE (31,32). Supporting this proposed mechanism, a study by Chiang et al. were point to that phenolic compounds are the major bioactive compounds found in extracts of *P. major* that exhibit potent anti-herpes and anti-adenoviral activities (33). Mahmood and Phipps, (33) and Chiang *et al.* (29) have been indicated that the antimicrobial activity of *P. major* against yeasts and bacteria related to their phenolic compounds, also traditionally been used for the treatment of skin bacterial infections (29,33).

The histopathological results were correlate with WCR results of PMLE group, histopathological section showed at 7th day were came in line with results of many studies by Patil *et al.* (25) and Sardari *et al.* (34) whom mentioned to the events of wound contraction begins approximately 4 to 5 days after initial injury and it is a cell-directed process: cell division is required, but collagen synthesis is not (25,34).

Wound edges move toward each other at a rate of approximately 0.6 to 0.75 mm/d. This process correlates in time with phenotypic morphogenesis of fibroblasts into myofibroblasts, as known myofibroblasts were appear in the wound approximately 4 to 6 days after injury and are identified by their contractile properties. In addition, Amini *et al.* (35) were recorded an improvement in wound contraction rate when used PMLE.in wound treatment and added a greatest response to the reduction of the area of wounds occurred between the 9th and 11th day in the PM treatment groups in addition to that investigate different the concentration of 20% and 50% which found not significantly difference between the experimental groups (35).

Medical dressings are essential devices in healthcare. According to the types and stages of wounds, dressings can be applied to their surface and promote healing (36,37). Using dressing in the current study may explained wound acceleration because dressing 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 Sarabahi (38), in addition P. spp. have a role in reduction of inflammation when used as a moist dressing. Other studies by Boateng *et al.* (39) and Mir *et al.* (40) explained the incidence of shortens in the inflammatory healing phase that associated with using of moist dressing and produces good cosmetic results.

Conclusion

High performance liquid chromatography (HPLC) evaluation of PMLE's phenolic and flavonoid contents confirmed that they play an effective role in accelerating contaminated wound healing thanks to their antioxidant and anti-inflammatory properties, which aid in the improvement of the (WCR) by encouraging early and additional fibroblast proliferation and angiogenesis compared to the control group.

Acknowledgments

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Conflict of interest

There is no conflict of interest.

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دراسة تجريبية لتأثير مستخلص أوراق لسان الحمل الكبير على التئام الجرح الاستئصالي الملوث في الأرانب

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الخلاصة

تم إجراء الدراسة الحالية لتقييم تأثير التطبيق الموضعي لمستخلص أوراق لسان الحمل الكبير وبتركيز ١٠% على عملية التئام الجرح الاستئصالي الملوث في الأرانب المحلية. تم استخدام عشرين من الأرانب الذكور البالغة. بعد أن تم تخدير الحيوانات تخدير عام، تم إحداث جرحين استئصاليين بمساحة (٢سم) في منطقة الصدر على الجانب الأيمن والأيسر (١جرح/جانب) لكل حيوان. الجروح في الجانب الأيمن تم علاجها بالغسل الموضعي بالمحلول الملحي المتعادل (مرة/يوم) قبل الضماد وهذه تعتبر المجموعة أ. بينما في المجموعة ب الجروح في الجانب الأيسر عولجت بوضع ١٠%من مستخلص أوراق لسان الحمل الكبير (مرة/يوم) موضعيا وبعدها ضمدت. تم تقييم تأثير مستخلص أوراق لسان الحمل الكبير على الالتئام من خلال الفحص العياني، تقلص الجرح (معدل تقلص الجرح) والفحص النسيجي المرضي. اثبت الفحص العياني إن مستخلص أوراق لسان الحمل الكبير قام بدور تسريع الالتئام عندما قورنت مع مجموعة السيطرة وهذه النتيجة تناغمت مع نتائج معدل تقلص الجرح والذي انعكس بوجود فروقات مهمة إحصائيا عند ٧، ١٤ و ٢١ يوماً بعد العلاج والذي سجل سرعة معدل التقلص في المجموعة أ مقارنة مع مجموعة ب. نتيجة الفحص النسجي المرضي اثبت زيادة مبكرة في تكوين الأوعية الدموية الجديدة, تكاثر الارومات الليفية، ترسب الكولاجين المتميز وتكون الظهارة المبكر في مجموعة ب مقارنة مع مجموعة أ. أثبتت هذه الدراسة أن التطبيق الموضعي ل ١٠% من مستخلص أوراق لسان الحمل الكبير له دور مأثر في تعجيل التئام الجروح الملوثة من خلال مكوناتها من الفينول والفلافينويد كما هو مثبت بفحص سائل فاصل للون عالي الكفاءة والتي عملت كمادة مضادة للأكسدة ومضادة للالتهاب والتي شجعت التكاثر المبكر والعالي للارومات الليفية والأوعية الدموية.

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