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EFFECTS OF TOPICAL APPLICATION OF PLANTAGO MAJOR LEAF ALCOHOLIC EXTRACT ON EXCISIONAL WOUND HEALING IN BALB/C MICE

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ABSTRACT

The aim of this study was to evaluate the effects of *Plantago* major L. leaves ethanol extract (pm) on excision wound healing in BALB/c mice.
Method: sixty male BALB/c mice (2.5 months of age) were divided into two experimental and three control groups (n=12).Under anesthesia, two circular excisional wounds (5mm diameter) were made on dorsal thoracic area of the mice skins by a disposable surgical punch. The experimental groups received topical cream of PM at the concentrations of 5% and 10% while control groups treated with nitrofurazone cream 1% (positive control), cold cream (negative control) or no treatment (sham control). On the days 4, 7, 10 and 14 after treatments, excisional biopsies were performed and wound healing was evaluated histopathologically.
Results: Compared to the negative control, Total inflammatory cells significantly decreased in both of the experimental groups at the days 4, 7 and 10. The proliferation phase markers including the number of fibroblast cells and granulation were significantly higher in experimental groups than negative control group. Assessment density of collagen fibers between groups showed that PM in a dose dependent manner significantly improved collagen synthesis.
Conclusion: The results of this study provide evidence that PM has beneficial effects on wound healing in BALB/c mice.

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Introduction

The skin plays a main role to protect against dehydration, bleeding and infection and regulate the body temperature (1, 2). Wound healing processes in three phases: inflammatory, proliferative and remodeling. Cutaneous wound healing occurs in stages that can be defined as a primary hemostatic event followed by an inflammatory phase that several types of inflammatory cells attend to the injury site; a proliferative phase in which new extracellular matrix (ECM) components are produced and involved in the migration and activity of the fibroblasts, appropriate blood supply (neovascularization) and permeability

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barrier (re-epithelization); and finally, remodeling phase reorganized new ECM and collagen structures to support the other cells associated with effective wound healing, as well as contracting the wound (3-7)

Traditional plants and their effective therapies have been explored in different studies and showed that herbal medicines have effective roles on wound healing by modulation of angiogenesis, proliferation of keratinocytes and anti-inflammatory effects (8, 9)

Plantago major (P. major.) is belongs to the Plantagina´ceae family. It has about 15 cm height with different size depending on the growth habitats. The oval shape leaves to elliptical with parallel venation. The fresh leaves are topically applied to infected chronic skin damage .The flowers are small and brownish-green (10, 11). The extract of P. major contains a powerful antioxidant compounds such as benzoic compound (vanillic acid), phenolic compounds (caffeic acid,chlorogenic acid, ferulic acid, p-coumaric acid), triterpenes (oleanolic acid, ursolic acid), flavonoids (baicalein, baicalin, luteolin) and iridoid glycoside (aucubin) (12, 13)**.** Moreover, the antioxidant activities of P. major are effective to wound healing process (14). The isolated pectin from the leaves of P. major contains homogalacturonan, that might be effective on wound-healing (15).Previous study showed that P.M leaves had various biological properties such as anti-inflammatory and antimicrobial to wound healing (16). However, a few studies have performed to clarify the wound healing effects of P. major. Therefore, the goal of this study was to elucidate the effects of P. major on the wound healing process.

Materials and Methods

All procedures were approved by the Medical Ethics Committee of Birjand University of Medical Sciences.

Experiment animals

In this study, 60 mature (2.5 months of age) male BALB/C mice were used. Mice were purchased from Pasteur Institute, Iran, and housed in the clean individual cages. Animals had free access to water and standard pellet diet. They were kept in standard environment of 12:12 h light-dark cycle, 22±1°C temperature; the air humidity was 60±5%.All animal procedures were conducted and approved in accordance with the guide for the laboratory animals care and usage of Birjand University of Medical Sciences, Birjand, Iran (Ethic code: Ir.Bums.1394.43).

Plant material and extract preparation

To prepare the alcoholic extract, the fresh leaves of P. major were collected from the suburb of Birjand, Iran in the spring of 2015 and the sample of this plant was confirmed by the resident botanist and a documented sample kept in the Herbarium at faculty of agriculture University of Birjand (Herbarium code: 1150). The fresh leaves washed with distilled water and dried in oven 50˚C for 2-3 days until fully dried. The leaves were ground to a fine texture form using a grinder. Weighing 50 gr of fine texture mixed with 500 cc alcohol (80%) and soaked in 40-50˚C for one day, then solution was removed by filtration using a mesh and filter funnel then rotatory vacuum evaporator extracted the filtered material. The extracts were submitted to lyophilization by a freeze-dryer to produce powdered forms of the extracts. The freeze-dried products were mixed with cold cream in concentration of 5% (PM 5%) and 10% (PM 10%) Eucerine, respectively.

Experiment design and wound healing evaluation

Mice were anesthetized with intraperitoneal (I.P.) injection of ketamine 70 mg/kg. Dorsal skin was shaved and two symmetrical full-thickness excisional wounds created besides the midline under anesthesia (at least 2 cm apart) with 5 mm disposable surgical punch (3, 17). The mice were randomly divided to five groups. Group I & II animals were treated with alcoholic extract of PM 5% and PM10% (treated groups), group III as the positive control, received nitrofurazone topical cream 1%; group IV were treated with cold cream (Eucerine) as the negative control group and group V that received no treatment (sham). All groups were treated twice daily at 8:00 am and 8:00pm. On days 4,7,10 and 14 after treatment, 3 animals from each group were sacrificed with an overdose of anesthetics and excisional wounds areas were removed for histopathological studies.

Histological Analysis

Excisional biopsies fixed in 5% formaldehyde solution were embedded in paraffin, sectioned at 5 μm and stained with H&E and specific staining of Trichrome Masson. Finally, slids assessed under light microscope by two independent pathologists in double-blind manner.

For each specimen, epidermal parameters (Re-epithelization) and dermal parameters (Inflammatory cells, Granulation and collagenisation) were evaluated.

Statistical analysis

The quantitative data were represented as mean ± standard error of the mean (SEM) or as percentages and frequency index was used for qualitative data. Oneway ANOVA test was used to compare in groups with each other and Tukey test was used in case of significant results. Data analysis was done using SPSS statistical software (ver.19). Results with $P < 0.05$ were considered as statistically significant. The quantitive parameters of inflammatory cells, epithelialization and granulation was measured by Image J software.

The areas of collagenisation were estimated by semi-quantitatively score of - to +++, following score system; - low, -/+ low to mild, +/- very mild, + mild, ++ moderate, +++ severe.

Results

Sample survey on day 4: The total number of inflammatory cells decreased significantly in treated groups of PM%10 (19.20±3.29) and PM%5 (24.80±3.73) and nitrofurazone (21±2.86) compared with negative control and sham groups (29.40±3.71 and 31.30±3.43, respectively) (P<0.05) (Table1).

The other parameters were not assessable on day 4.

Sample survey on day 7: The findings of day 7 based on inflammatory cells, demonstrated that, PM%10(39±3.65), PM5% (44.9±4.17) and nitrofurazone (41.6±3.89) significantly had a better response to decrease inflammation in comparison with the negative control and sham groups (52.4±4.03 and 54.5±3.95, respectively) (P<0.05) (Table2).No differences in the total number of inflammatory cells was observed in the PM5%, PM10% and nitrofurazone groups (P<0.05).

In this day, the area of granulation was significantly increased in PM10% (1453701.5±110270.85μm^2) and PM5% (1288840.9±103551.49μm^2) compared to negative and sham control groups (994317.9±113612.69μm^2) and (1056732.8±104813.87μm^2, respectively)(p<0.05)(Table1).

Our findings showed that the mean of fibroblast cells was also increased in treated groups with PM10 %(28.1±1.85) and PM5% (25.4±2.67) compared to negative and sham control groups (22.1±2.37 and 20.3±1.63, respectively). (P < 0.05) (Table1).

Furthermore the mean of fibroblast cells in PM treated groups was higher than nitrofurazone group (24.6±2.95).

Collagenisation scores were significantly greater in treated groups of PM10% (+) and PM5% (+) compared to the negative and sham control groups (-) (Table1). There is no significant changes in the total density of collagen between PM5% and PM10% treated groups with nitrofurazone group (Table1).

Sample survey on day 10: (Fig 1) showed that Total inflammatory cells infiltration, thickness of epithelialization and granulation tissues on day 10. The findings showed that, doseses of PM10% (20±3.46), PM5% (24.1±4.62) and nitrofurazone (22.2±4.0420) caused a significant reduction in oedema compared with the negative and sham control groups (33.1±2.88 and 30.8±4.36, respectively). (P < 0.05) (Table1).

The rate of granulation tissue formation was significantly different between groups which received

Treatments PM10% (2119950±120643.03μm^2) and PM 5% (1989215.1±117523.7μm^2) comparison to the negative and sham control groups (1677652.8±114694.84μm^2 and 1561904.1±126046.83μm^2, respectively) (p<0.05). The mean of granulation in the PM10% was significantly higher than nitrofurazone group (1873822.3±107346.8μm^2) (p<0.01) (Table1).

The mean of fibroblast cells were significantly increased in treated groups of PM10% (52.4±3.53) and PM5% (49.9±3.14) compared to the negative and sham control groups (45±2.05)(44.2±2.61) (p<0.05)(Table1).

The number of fibroblast cells in PM10% treated groups was significantly higher than nitrofurazone group (48.3±2.82) (p<0.05).

The score of collagen synthesis in groups of PM10% (++) and PM5% (++) was higher than negative and sham control groups (+/-) an increase was observed in collagenisation of PM10% and PM5% groups (++) compared to nitrofurazone group (+) (Table1).

Sample survey on day 14: The granulation was significantly increased in treated groups of PM 10% (1990771±97587.32μm^2) and PM5% (1818778±98804.93μm^2) compared to the negative and sham control groups (1439550±109250.98μm^2 and1533875±104634.83μm^2, respectively) (p<0.05). The mean of granulation in PM10% treated group was significantly higher than nitrofurazone (1683935±90837.16.8μm^2) (p<0.05) (Table1).

There was a significant increase of fibroblast cells in treated groups with PM10% (38.6±2.41) and PM5% (36.2±2.39) negative and sham control groups(29±2.78 and 30.1±2.42 ; respectively) (p<0.05).

Also, the number of fibroblast cells in PM10% treated groups were significantly more than nitrofurazone group (34.4±2.87) (p<0.05) (Table1).

The score of collagenisation was found to be higher in treated groups of PM10% (+++) and PM5% (+++) compared to the negative and sham control groups (+) (Table1). There is no changes in the total density of collagen between PM5%, PM10% and nitrofurazone groups (+++) (Table1).

Table 1. Effect of topical Plantago major leaf alcoholic extract on various epidermal and dermal parameters on wound healing

	days	PM10%	PM5%	NIT	EU	SHAM
The total number of inflammatory cells	4	19/20±3/29 *	24/80±3/73 *	21±2/86 *	29/40±3/71	31/30±3/43
	7	39±3/65 *	44/9±4/17 *	41/6±3/89 *	52/4±4/03	54/5±3/95
	10	20±3/46 *	24/1±4/62 *	22/2±4/04 *	30/8±4/36	33/1±2/88
Granulation μm^2	7	1453701/5± 110270/858**	1288840/9± 103551/49 *	1167868/6± 103597/13	1056732/8± 104813/87	994317/9± 113612/69
	10	2119950±	1989215/1±	1873822/3±	1677652/8±	1561904/1±

		120643/03 **	117523/7 *	107346/18 *	114694/84	126046/83
	14	1090771± 97587/32 **	1818778± 98804/93 *	1683935± 90837/16 *	1533875± 104634/83	1439550± 109250/98
Fibroblast cells	7	28/1±1/85 **	25/4±2/67 *	24/6±2/95	22/1±2/37	20/3±1/63
	10	52/4±3/53 **	49/9±3/14 *	48/3±2/62	45±2/05	44/2±2/61
	14	38/6±2/41 **	36/2±2/39 *	34/4±2/87 *	30/1±2/42	29±2/78
Thetotal collagen	**7**	+	+	+	-	-
	10	++	++	+	+/-	+/-
	14	+++	+++	+++	+	+

Mean ± SD Anova and Tukey tests , - low, +/- very mild, + mild, ++ moderate, +++ severe

* P<0.05, ** P<0.01

Plantago major 5%(PM5%)

Plantago major 10%(PM10%)

Plantagolanceolata5%(PL5%)

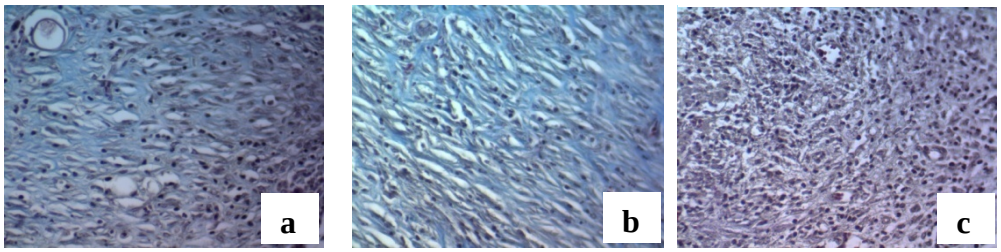
Plantagolanceolata10%(PL10%)

Nitrofurazone(NIT)

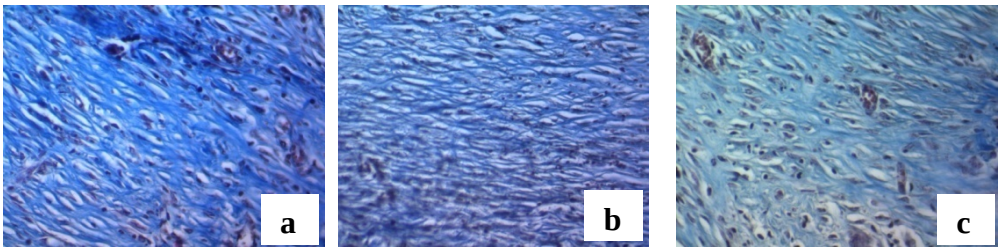
Eucerine(EU)

Sham

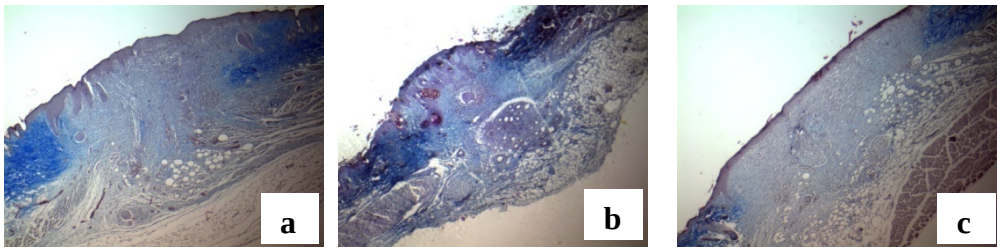
(i) Inflamatory cells



(ii) collagenisation



(iii) Granulation



Figur1. (i-iii): (i), Total inflammatory cells infiltration in a(pm10%), b(pm5%), c(sham). Trichrom stain. Magnification of 400x in 257/55x334/65 µm2 on day 10 (ii), collagenisation in a(pm10%), b(pm5%), c(sham). Trichrom stain. Magnification of 400x in 257/55x334/65 µm2 on day 10 (iii), The area of granulation in a(pm10%), b(pm5%), c(sham).Trichrom stain. Magnification of 400x in 3204/12x2401/34µm2 on day 10

Discussion

Cutaneous wound healing is a dynamic and interactive process of cellular and biochemical events involving three phases of inflammation, tissue formation and tissue remodeling. The inflammatory response is composed of vascular permeability response and a leukocyte infiltration. PMN are usually the first cell followed by mononuclear leukocytes. Infiltrating neutrophils cleans the wounded area from foreign particles. The monocyte-and macrophage are certainly necessary for organizing new tissue formation in the wounds (18, 19). Polyphenols extracted from leaves of P. major have anti-inflammatory, antioxidant and antiviral effects on wound healing (20).In the beginning of this study (on day 4), the findings showed that inflammation and infiltration of inflammatory cells in the treated groups were reduced compared to the control groups. Also, the results were similar to the findings related to days 7 and 10. Pouresmaeil et al. (2003) demonstrated the anti-inflammatory effects of P. major aqueous extract on inflammation from chemotherapy and suggested that the aqueous extract of P. major leaves could be helpful in the treatment of chemotherapy-induced stomatitis (21). Samuelsen et al. reported that isolated compounds from P. major seeds and leaves have been used as a wound healing remedy. The leaves also contain compounds with anti-inflammatory activities, such as plantamajoside, baicalein, hispidulin, aucubin, ursolic acid and oleanolic acid. Plantamajosid has been also known to have some antibacterial activities. Moreover, a number of flavonoids extracted from P. mayor are showed to have antioxidant properties with the free radical scavenging activities. According to the literature, as a natural source of antioxidants, P. major applied for treatment of different diseases including the diseases related to the skin, respiratory organs, digestive organs, reproduction, and the circulation, against cancer, for pain relief and against infections (22)**.**

Granulation tissue formation is categorized as the fibroplasias and neovascularization. Fibroplasia consists of granulation tissue components include fibroblasts and macrophages and angiogenesis and fibroblasts construct new ECM which is necessary to support wound repair. Blood vessels carry oxygen and nutrients to newly forming tissue (23). On days 7, 10 and 14, the results of the study showed that the mean number of fibroblast cells and granulation tissue in treated groups (PM%10, PM%5) and Nitrofurazone were significantly higher.On day 14, the complete wound closure observed in P. major treated wounds.

In the studies of Krasnov et al in 2002 on the effect of low doses of the novel regulatory plant proteins showed that 100 proteins obtained from plantain influences proliferation of human fibroblasts in vitro.(24)

Amini et al. determined the effects of 20% and 50% P. major liquid extracts on healing of burn wound. According to their results, 50% P. major had the better effects compared to 20% and the best time of impacts were seen on day 21. Furthermore, increased in angiogenesis process was reported in the treated groups with P. major extract compare to other control groups (25). Mahmood et al evaluated the wound healing activities of P. major leaf extract in rats. Queued leaf extract of P. major with 5% and 10% doses were used. Their results showed that treated groups with P. major extract healed earlier and faster (13)**.**

Maturation Phase is a reorganization of collagen fibers and increase in tensile strength. The majority of the type III collagen fibers synthesis in wound healing area and after a 5-day replaced by a high rate of type I collagen synthesis (19). Increasing of types I and III collagen deposition occurs between the 7 and 14 days (26). On days 10 and 14, the density of collagen fibers in treatment groups (PM%10 and PM%5) was increased. In vitro experiments showed that high extracellular glucose could stimulate the productions of type III collagen and fibronectin by mesangial cells. Probably due to the hexon combinations in extract of Plantago, it had positive significant effects on wound healing area by increasing the fibroblast cells, collagen fibers and tensile strength.

Conclusion

The results of the present study support the beneficial effects of P. major on wound healing process and its potential clinical applications. The authors of this study recommend the evaluation of all effective ingredients of this plant on wound healing process and using the most effective compounds in wound healing topical treatments.

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