Original Article

Exploring the Role of Phenolic Extract As an Ointment Dosage Form in Inducing Wound Healing in Mice

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Abstract

Background: Wound healing is a multi-phase and well-organized dynamic process involving the coordinated use of numerous cell types and biological processes to repair damaged tissue, which relies on many inter-related factors. The failure of traditional healing protocols in many cases of acute wound healing that may progress to gangrene or lead to chronic wounds and amputation provides a strong stimulus to discover a neoteric, relatively long-term, safe, and effective treatment. Antioxidant, anti-inflammatory, and anti-microbial mechanisms are used. As is widely known, plant secondary metabolites such as phenolic compounds, besides their beneficial impact on the plant host, can be effective for humans in treating a variety of disorders. The most common properties of polyphenols—antioxidant, anti-inflammatory, and antimicrobial—indicate that they deserve recognition in natural medicine and may be highly effective in the treatment of various skin problems. Those three mentioned properties constitute the main potential mechanisms of action against various skin disorders.

Aim: To compare the effects of phenolic extract from Iraqi Petroselinum crispum to β-sitosterol on wound size reduction, histological outcome, and biomarkers of tumor necrosis factor-alpha, as well as identify the most likely mechanism of action in experimentally induced acute wounds in mice.

Methodology: Phenolic compounds are extracted from Iraqi petroselinum crispum plants by universal methanol (80%) solvent, followed by fractionation by ethyl acetate to obtain phenolic compounds. The dose of phenolic compound will be (13,22%) w/w of extract and the phenolic extract powder mixed with Vaseline to obtain the ointment.

In vivo study: Fifty male albino mice were enrolled in this study, and they were divided into five groups (N = 10/group). Group I served as normal control. Group II: served as induced control which has received a petrolatum base only (negative control).Group III: Mice with induced wounds were treated topically with β-sitosterol ointment (0.25% w/w) as a standard drug (positive control). Group IV and V: mice with induced wounds were treated topically with a phenolic extract of Petroselinum crispum ointment (13% and 22%w/w). respectively These products were applied twice daily for 10 consecutive days.

Results: Phenolic extract ointment produced a highly significant reduction in wound size in comparison with petrolatum base ($P \leq 0.001$).

In this histological study, re-epithelization and angiogenesis scores of all treatment groups showed a significant increase in comparison with the petrolatum base group ($P \leq 0.05$), but in collagen scores, the significant increase just occurred with the highest concentration of phenolic extract ointment (22% w/w) in comparison with the petrolatum base group ($P \leq 0.05$).

In cytokine outcome at the end of the experiment, petroselinum crispum (13%) produced a significant decrease in tumor necrosis factor-alpha in comparison with the β-sitosterol group ($Pc \leq 0.05$).

Conclusion: According to the present findings, we can conclude that phenolic extract from petroselinum crispum ointment is more efficient than petrolatum base and comparable in efficacy to β-sitosterol ointment in accelerating wound healing.

Keywords: Attention, concentration, referee.

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Introduction

Wound healing is a crucial homeostatic mechanism that entails several coordinated and multi-phase processes, such as (1) the processes of hemostasis, inflammation, proliferation, and remodeling (2,3). Tissue damage occurs during acute inflammation, which is afterwards resolved. Whereas with chronic inflammation, damage and repair occur over an extended period of time. (4). Neutrophils, an example of an inflammatory cell, phagocyte invading pathogens, remove waste and debris, and also promote angiogenesis, which may accelerate the recruitment of inflammatory cells and the subsequent laying down of extracellular matrix to repair tissue injury. (5). Long-lasting inflammation may cause tissue injury, and aberrant or insufficient healing may result in fibrosis and poorly ordered matrix deposits that alter the normal architecture of the tissue. (6).

The function of phenolic extract in wound healing

According to in vitro and in vivo studies, polyphenols' anti-inflammatory, antioxidant, antibacterial, and angiogenic activities have a positive impact on the healing of skin lesions. These compounds make up an intriguing therapeutic strategy for wound healing, whether used alone, in conjunction with other treatments, or as phenolic extracts, which are abundant in different polyphenols that may have synergistic effects. Clinical trials are required to verify these results in people and to comprehend the mechanisms behind the activities of these drugs. (7)., Phenolics enhance collagen synthesis in fibroblasts and improve antioxidant defense and enzyme activity in cells by over-expressing Nrf2. Dermal damage is prevented by topically applying certain extracts. These findings suggest that phenolic chemicals may aid in fibroblast activity, speed up the healing process, and prevent UV-induced photoaging. protection.(8).

The effects of phenolic extract on inflammation

The inflammatory reaction starts happening right away after the damage. By triggering a local inflammatory response that attracts diverse inflammatory cell types to the site of the wound, the innate immune system is triggered. (9).The potent antioxidant effects of phenolic compounds are mediated by scavenging free radicals like ROS, suppressing the formation of ROS by inhibiting specific enzymes or chelating trace minerals required for their production, and finally by up-regulating or protecting the antioxidant defense system (10).

The effect of phenol on angiogenesis

Polyphenols are a class of natural compounds whose potential as antioxidants, anti-inflammatory, and anti-angiogenesis has been reported in many pathological conditions (11).

Materials and methods

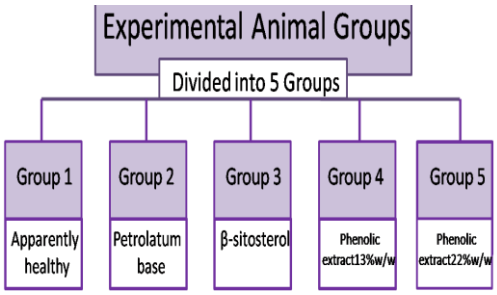
Materials

Phenolic extract powder was purchased from Al-Razi Center for Alternative Medicine.B-Sitosterol ointment was obtained from Philadelphia/Jordan, formalin (MW = 30.3) was provided by SOLVOCHEM/UK, castor oil was obtained from Jayant Agro-organics LTD, India, and petrolatum base was provided by Regent chemicals, India.

Preparation of topical phenolic extract ointment from Petroselium crispum

Since petroselium is not available in a topical dosage form,

I buy from the market 1 kelo of fresh petroselinum crispum and then wait for it to dry (the aerial part). Then I blend it in a blender machine to get a powder of about 200g. It was defatted with hexane (1400 ml). The defatted plant material was further extracted with ethanol (1500 ml) using a soxhlet extractor. The ethanolic extract was concentrated by evaporation under reduced pressure using a rotary evaporator. Then distilled water (35ml) was added to the ethanol extract and the extract portion with ethyl acetate (50 ml) was allowed to stand over night. The phenolic compounds were extracted from the Petroselinum crispum plant. The extraction was by universal methanol (80%) solvent, followed by fractionation by ethyl acetate to obtain phenolic compound powder. Afterwards, the powder was mixed well with petrolatum base by the incorporation method to achieve the required weight of 100 gm. In the incorporation method, a small amount of petrolatum base was mixed carefully with a small volume of phenol powder for about 5 minutes until a clear, homogenized ointment was obtained. The prepared phenolic extract ointment was then transferred into clean and sterile plastic cups, which were closed tightly and stored at normal room temperature.



Experimental animal groups

Fifty mice were involved in the experiment. Each group contained ten mice, which were selected randomly from the total mice involved in the experiment, as illustrated in Figure 1.

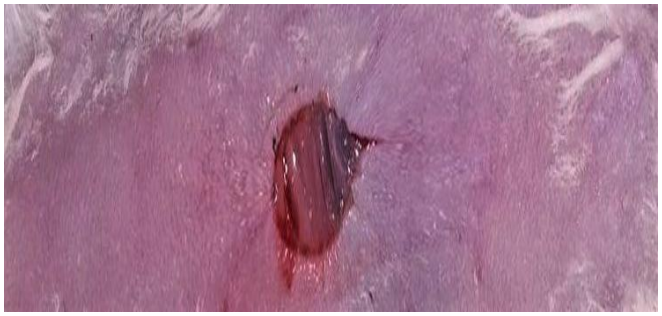


Figure 1. Experimental Animal Groups

Model of a murine wound

Mice were anesthetized by intraperitoneal injection of "ketamine (100mg/kg)/xylazine (10mg/kg)". Afterwards, the back-skin hair was shaved using shaving cream, and a 1-cm full-thickness excisional wound was then made on the back of each mouse using a sterile 10-mm biopsy punch (12) as shown in Figure 2.

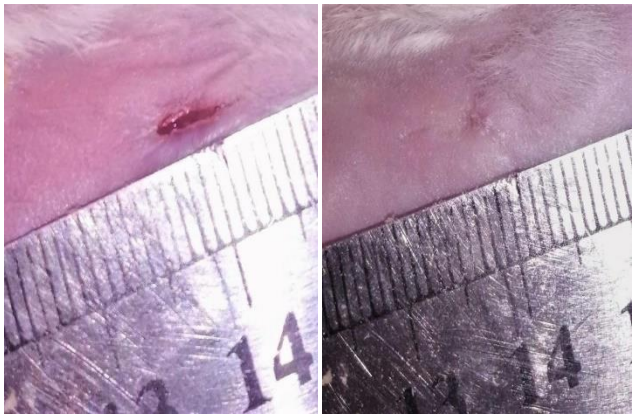


Figure 2. Experimental Wound induction by punch biopsy

Wounds size Measurement

The wound size areas for 6 mice from each group were randomly selected and measured by a ruler from edge to edge on the 5th and 10th days of the experiment. The difference in wound size reduction between the experimental groups was compared using the following equation:

% Wound Closure = (primary wound area-end wound area)/100% of the primary wound area

The primary wound area (on day 0) was defined as 1 cm (13). The wounds on the tenth day were carefully dissected by a sharp, sterile surgical blade. Tissues were collected without folding by forceps, which were stabilized in 10% buffered formalin solution and stocked into a sterile plastic container for storage, ready for embedding in paraffin wax for histopathological and immunological study.

Assessment of the histopathological changes of tissue sections (H & E stains)

were used for the staining of paraffin wax sections (14). The skin histopathological changes for each mouse were scored (15). The amounts of collagen, angiogenesis, and re-epithelization scores were made and evaluated as: 0 = absent or a few, 1 = moderate presence, and 2 = plenty.

ELISA kits for the detection of TNF-α

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat TNF-α. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat TNF-α, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat TNF-α. You can calculate the concentration of Rat TNF-α in the samples by comparing the OD of the samples to the standard curve.

Results and discussion

Preparation of topical phenolic extract from petroselium crispum ointment

Since phenolic extract from petroselinum crispum is not yet available in a topical dosage form, the challenge of developing a successful semi-solid topical ointment formulation has been more promising than other topical dosage forms taking into account the properties of the active ingredient (phenol) which is water insoluble and the attractive properties of the ointment base (petrolatum) including its ability to cover the site of application for a long time and it will act as a barrier that prevents foreign substances and microorganisms from penetration into the tissue through the wound. In addition, the ointment base increases the hydration of the skin due to its oil nature which prevents moisture evaporation leading to an increase in drug penetration (16). Furthermore, the prepared phenolic extract ointment will be in a similar dosage form when compared to the positive control β-sitosterol ointment, since both of them have been used in an ointment dosage form. This will minimize any influence of the dosage form type on the wound healing process.

Wound size reduction:

The data obtained has revealed a highly significant reduction in the wound size of the treatment groups as compared with the induced control group (Pa≤0.001),but there were no significant differences in comparisons between the treatment groups (Pb, Pc > 0.05) as shown in Table 1 and figure 3. This demonstrates the efficacy of phenolic extract ointment in reducing wound size and hastening wound closure.It has been demonstrated that phenolic molecules, particularly

flavonoids, are powerful antioxidants that are superior to carotenoids, vitamin C, and vitamin E (17).

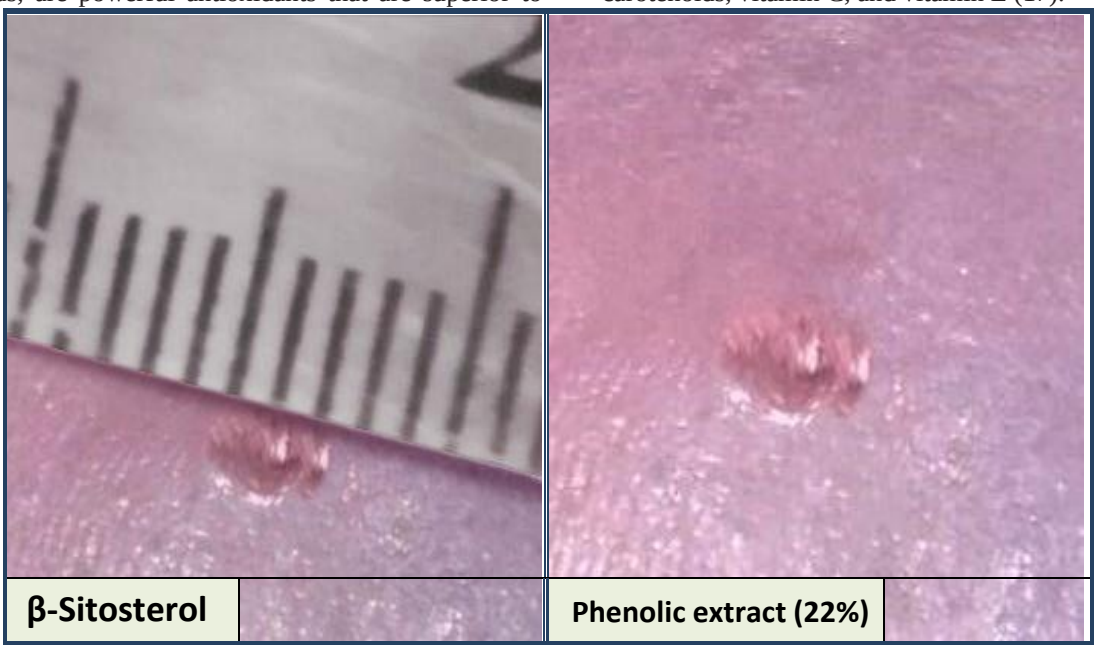


Figure 3: Wound images on the tenth day, showed completely wound closure in treatment groups, but incomplete wound closure in the petrolatum base group.

Table 1: Comparison of wound size reduction in milli meter between induced control and each other treatment groups and among other treatment groups by un paired t-test.

Days		**Vaseline N=6**	**b-Sitosterol N=6**	**Phenolic extracts from *Petroselinum crispum* 13 mg N=6**	**Phenolic extracts from *Petroselinum crispum* 22 mg N=6**
Day5	Mean±SE	32.00+1.67	48.17+0.79	47.67+0.92	48.17+0.79
	Median	31.50	48.50	48.00	48.50
	P-value*a*		**0.000**	**0.000**	**0.000**
	P-value*b*			**0.689**	**1.000**
	P-value*c*				**0.689**
Day10	Mean±SE	73.50+1.61	97.33 +1.31	97.33 +1.31	97.67 +1.20
	Median	74.00	98.50	98.50	99.00
	P-value*a*		**0.000**	**0.000**	**0.000**
	P-value*b*			**1.000**	**0.885**
	P-value*c*				**0.885**

- Data represented as Mean±Standard error(SE).
- N=number of animals from each group tested per day.
- (a) Comparison between the petrolatum base (vaseline) group and each treatment group, (b) Comparison between β-Sitosterol and both petroselinum crispum (13% and 22% w/w). (c) Comparison between phenolic extracts from Petroselinum crispum (13% and 22% w/w).

Histopathological scores on the tenth day

Re-epithelization score

There was a significantly lower in the petrolatum base (Vaseline) group (Pa≤ 0.05), but in β-sitosterol and both phenolic extracts from petroselinum doses (13 and 22 %w/w) groups, there were no significant differences with the apparently healthy group (Pa > 0.05) as shown in Table 2. β-sitosterol and both phenolic extracts from petroselinum doses (13 and 22% w/w) groups have a significantly increased score as compared to the petrolatum base (Vaseline) group (pb≤0.05). In a comparison of β-sitosterol with both phenolic extracts from petroselinum doses (13 and 22 %w/w) groups, on the tenth day, there were no significant

differences between them (Pc > 0.05) as illustrated in Table 2. A wound is covered in fresh epithelium through the process of re-epithelialization. The cellular and molecular processes that begin, continue, and complete epithelialization are necessary for successful wound closure (18).

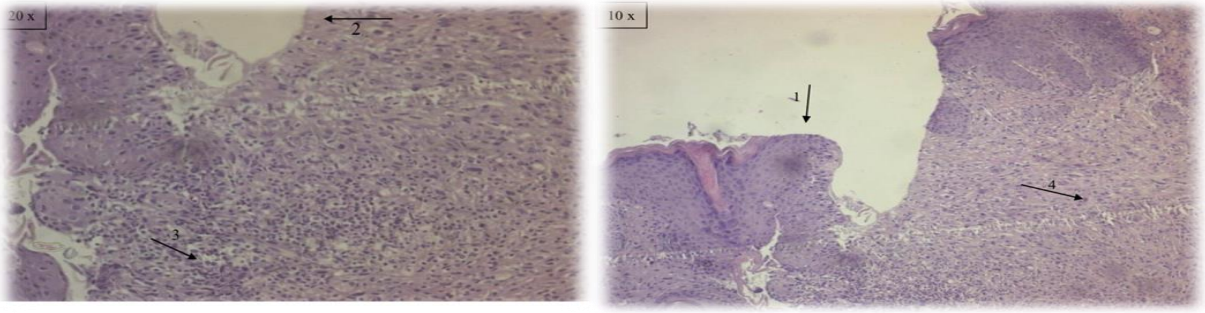
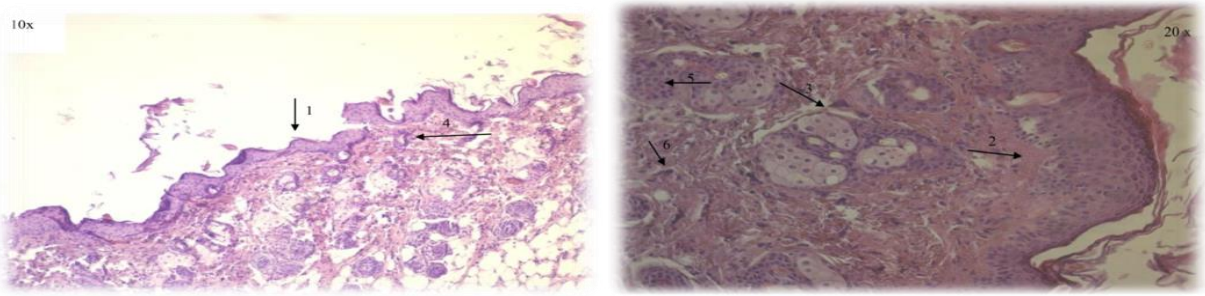
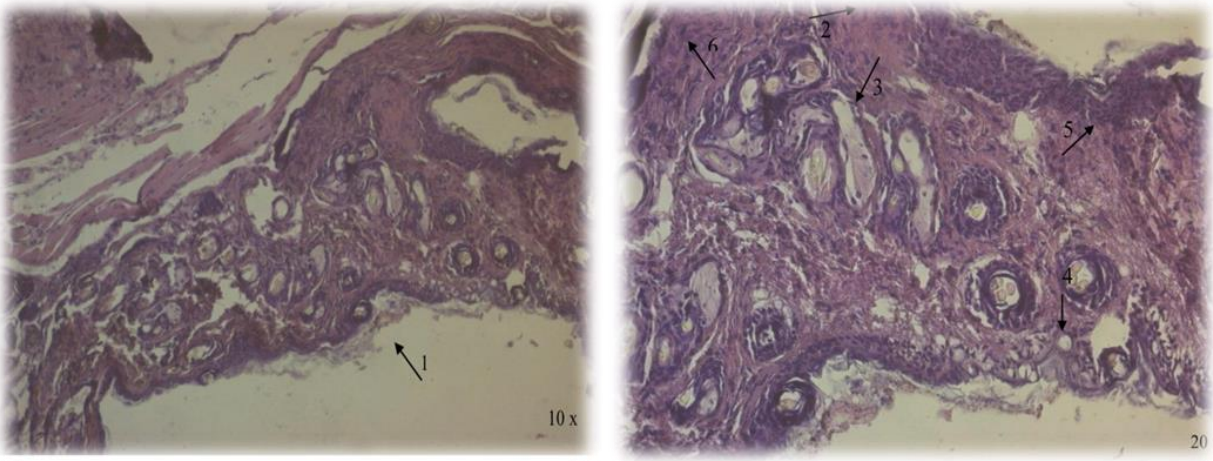


Figure (4A) Cross-section of wound tissue at tenth day for vasaline group;Showed high presence of inflammatory cells and fibroblasts but mild collagen, angiogenesis, and re-epithelization on microscopical examination (H&E staining) (10x)and (20x). 1.Re-epithelialization. 2.Collagen. 3.Infllamatory cell. 4.Fibroblast



Figure(4B) Cross-section of wound tissue at tenth day for β-sitosterol group; Showed the presence of few inflammatory cells, fibroblasts ,high angiogenesis, collagen and completed re-epithelization on microscopical examination (H&E staining) (10x)and (20x). 1.Re-epithelialization. 2.Collagen. 3.Hair follicle. 4.Blood vessels. 5.Infllamatory cell.6.Fibroblast.



Figure(4C) : Cross-section of wound tissue at tenth day for petroselnium crispum 13 % group;Showed the presence of few inflammatory cells, fibroblasts, high angiogenesis, dense collagen and completed re-epithelization on microscopical examination (H&E staining) (10x) and(20x). 1.Re-epithelialization. 2.Collagen. 3.Hair follicle. 4.Blood vessels. 5.Infllamatory cell.6.Fibroblast.

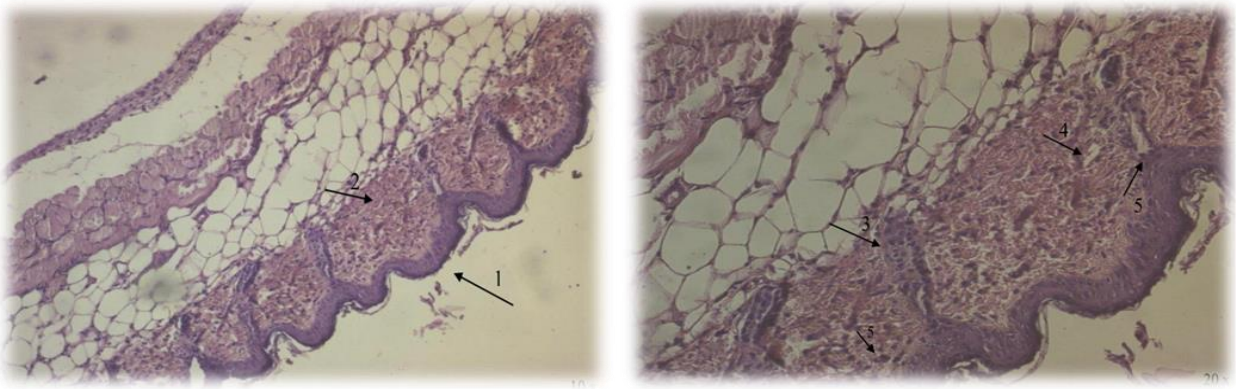


Figure (4D): Cross-section of wound tissue at tenth day for petroselinum crispum 22 % group; Showed the presence of few inflammatory cells, fibroblasts, high angiogenesis, very dense collagen and completedre-epithelization on microscopical examination (H&Estaining)(10x)and(20x). 1.Re-epithelialization. 2.Collagen. 3.Hair follicle. 4.Blood vessels. 5.Infllamatory cell. 6.Fibroblast.

Table(2): Comparisons of histopathology at tenth day between healthy,induced control,and each other treatment groups and among each treatment group byMann Whitney test.

Histopathology		**Control N=6**	**Vaseline=6**	**β-SitosterolN =6**	**Phenolic extracts from *Petroselinum crispum* 13 %N=6**	**Phenolic extracts from *Petroselinum crispum* 22 %N=6**
Collagen	Mean±SE	2.00+0.00	1.33 +0.21	2.00 +0.00	1.67+0.21	2.00 +0.00
	Median	2.00	1.00	2.00	2.00	2.00
	P-value *a*		**0.065**	**1.000**	**0.394**	**1.000**
	P-value *b*			**0.065**	**0.394**	**0.065**
	P-value *c*				**0.394**	**1.000**
	Pvalue *d*					**0.394**
Angiogenesis	Mean±SE	2.0 0+0.00	1.16+0.17	1.67+0.21	2.00 +0.00	2.00 +0.00
	Median	2.00	1.00	2.00	2.00	2.00
	P-value *a*		**0.015**	**0.394**	**1.000**	**0.015**
	P-value *b*			**0.180**	**0.015**	**0.015**
	P-value *c*				**0.394**	**0.394**
	P-value *d*					**1.000**
Re-epithilization	Mean±SE	2.00+0.00	1.16+0.17	2.00 +0.00	2.00 +0.00	2.00 +0.00
	Median	2.00	1.00	2.00	2.00	2.00
	P-value *a*		**0.015**	**1.000**	**1.000**	**1.000**
	P-value *b*			**0.015**	**0.015**	**0.015**
	P-value *c*				**1.000**	**1.000**
	P-value *d*					**1.000**

- Data represented as Mean±Standard error(SE).
- N=number of animals from each group tested per day.
- (a)Comparison between healthy and all other groups.

- (b)Comparison between petrolatum base and treatment groups.
- (c) Comparison between β-sitosterol and both phenolic extract from petroselinum crispum .
- (d) Comparison between both phenolic extract from petroselinum crispum (13 and 22 %w/w).

Angiogenesis score

Apparently healthy group when compared with other groups, at the tenth day, petrolatum base (Vaseline) and Phenolic extracts from petroselinum crispum 22 % group there was significant difference (Pa≤0.05), but in β-sitosterol and Phenolic extracts from petroselium 13 % groups found no significant differences with an apparently healthy group (Pa>0.05) as shown in table 2. β-sitosterol and both Phenolic extracts from petroselinum doses (13 and 22 %w/w)groups scores were no significant difference with β-sitosterol (pb>0.05) and significant difference with both Phenolic extracts from petroselinum doses (13 and 22 %w/w) as shown in table 2.and Figure4B. Wound healing is a complex process that involves huge number of cell types that act in a specific sequence to repair tissue ,One highly observable part of normal healing is the creation of a new capillary bed via angiogenesis. In skin wounds, angiogenesis proceeds by the creation of a dense but poorly organized capillary bed that is eventually trimmed back to normal density and capillary architecture ,It has long been assumed that a high level of capillary growth is essential for optimal healing, but a recent study has challenged that notion, The latest research indicates that normally healed wounds display an excessively strong and essentially dysfunctional angiogenic response that may negatively impact repair success(19).

Collagen score

Apparently healthy skin when compared with other groups, on the tenth day, there was no significant deference in all groups (Pa>0.05) as shown in Table (2).

In a comparison of the Vaseline group with β-sitosterol and both phenolic extracts from petroselinm (13 and 22%) groups, on the tenth day there was no significant difference in comparison between Vaseline and β –sitosterol and both doses of the phenolic extracts from petroselinum group (Pb > 0.05).

There were no significant differences in comparisons between β-sitosterol and both phenolic extracts from petroselinum (13 and 22%) groups (Pc and Pd > 0.05) on the tenth day, as shown in Tables (2) and Figure 4C. Collagen plays a central role in wound healing as it is a principal component of connective tissue that provides a structural framework, strength, and a milieu for the regenerating tissue. Collagen is produced by fibroblasts and helps the wound gain tensile strength during repair (20).

ELISA expression of TNF-alpha scores at the tenth day

Apparently healthy skin group when compared with other groups At the tenth day, those were significant increments in all other groups(Pa≤0.05). In a comparison of β-sitosterol and both petroselinum (13 and 22 %w/w) with Vaseline group, on the tenth days, there were a significant increase in β-sitosterol and petroselinum 22 % groups (Pb≤0.05),except in the petroselium 13 mg at the tenth day was statistically no significant increment (Pb>0.05) as shown in Tables (3). There were no significant differences in comparisons between the β-sitosterol and petroselinum 13 mg groups (Pc >0.05)but significant deference in petroselinum 22% (Pc≤0.05). In comparison between petroselinum 13 mg and 22 mg there were significant deference in petroselinum 22 % (Pd≤0.05).TNF- α is promptly released by fibroblasts, keratinocytes, and vascular endothelial cells in the injured area, which starts the inflammatory phase by attracting inflammatory leukocytes to the injured tissues. Major cellular sources of TNF- α are converted into recruited neutrophils and macrophages during the inflammatory phase, and this conversion results in a positive amplification circuit for extending the inflammatory reactions. Additionally, TNF α - controls the production of extracellular matrix proteins and matrix metalloproteinases, which are crucial for the repair of damaged tissues, as well as the activity of fibroblasts, vascular endothelial cells, and keratinocytes(21).

Table(3) Comparison of cytokins parameters(TNF-α) levels on the tenth day between healthy, induced control, and each β-sitosterol and both petroselium crispum(13 and 22%w/w) by Mann Whitney test.

Parameter		**Control N=6**	**VaselineN =6**	**β-SitosterolN =6**	**Phenolic extracts from *Petroselium crispum* 13 %N=6**	**Phenolic extracts from *Petroselium crispum* 22 %N=6**
TNF-α (pg/ml)	Mean±SE	54.83 +26.68	0.65+0.09	0.2+0.04	0.36+0.09	0.07+0.01
	Median	39.43	0.56	0.2	0.37	0.07
	P-value *a*		**0.009**	**0.002**	**0.002**	**0.002**
	P-value *b*			**0.002**	**0.240**	**0.002**

	P-value *c*				**0.180**	**0.002**
	P-value *d*					**0.002**

- Data represented as Mean±Standard error(SE).
- N= number of animals from each group tested per day.
- (a) Comparison between healthy and all other groups.
- (b) Comparison between petrolatum base and β-sitosterol, phenolic extract from petroselinum crispum (13 and 22 %w/w).
- (c) Comparison between β-sitosterol and both petroselium crispum(13% and22%w/w) (d) Comparison between phenolic extract from petroselium crispum 13% and 22% w/w)
- TNF-α: tissue necrosis factor-alpha.

Conclusion

In all parameter scores that have been measured, topical application of phenolic extract ointment from Petroselinum crispum twice daily for 10 days on induced wound appears to be more effective in accelerating wound healing than petrolatum base and comparable in efficacy to β-sitosterol ointment.

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Interest conflict

None

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