Research Article

Preparation and evaluation of Hesperidin ointment for burn wound healing potential

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Received: 10 June 2024 Revised: 21 August 2024 Accepted: 28 August 2024

Abstract

Objective: Wound healing process consists of different phases such as granulation, collagenation, collagen maturation and scar maturation are some of the many phases, which are independent to each other. Objective of present study was to prepare and evaluate Hesperidin ointment for burn wound healing effect. **Material and methods**: Hesperidin ointment (HO, 2.5 and 5%w/w) was prepared by using simple ointment base B.P. Ointment prepared by fusion method and evaluated using different evaluation parameters such as pH, physical appearance, diffusibility, extrudibility. The prepared hesperidin ointment formulations were evaluated for effect on full thickness burn wound model in rats. **Results**: Results were shown that wound contraction from days 12 to 18, was found significantly (P<0.05) faster in rats treated with hesperidin ointment and comparable with marketed formulation, 5 % w/w Povidone Iodine Ointment USP. We point out that burn wounds treated with ointment base alone did not achieve full-closure by day 20, while wounds treated with HO 5%w/w and Reference ointment groups was found complete healing in 18 days. In addition, 2.5 % w/w HO ointment treated groups of animals were found wound closure occurred in 20 days. The hydroxyproline content in animals treated with HO 5%w/w, HO 2.5% w/w and reference ointment was found significantly (P<0.05) greater than control group of animals. Over the 20-day treatment period, the protein content of wounds treatment groups with HO 5%w/w (72.33±2.85) was found significantly (P< 0.05) increased than control group of animals. **Conclusion**: In conclusion, Hesperidin having strong antioxidant, healing effect and anti-inflammatory potential as already reported by various researchers. These properties of hesperidin may supports in present study of hesperidin ointment for burn wound healing effect.

Keywords: Hesperidin, burn wound, ointment, Povidone Iodine Ointment, antioxidant

Introduction

Wound healing is a complex dynamic process. Wound environment changes with the changing heath status of the individual. The knowledge of the physiology of the normal wound healing trajectory through the phases of hemostasis, inflammation, granulation and maturation provides a framework for an understanding of the basic principles of wound healing (Kerstein, 1997).

Burn injury to the skin and deeper tissues may be caused by hot liquids and solids, flames, radiant heat, caustic chemicals, electricity and radiation. Different methods are employed for the treatment of burn injuries depending upon the cause of burn (Sultana et al., 2004).

Hesperidin belongs to the flavanones subclass of flavonoids and is found primarily in citrus fruits, such as oranges, grapefruits, lemons, and tangerines. Research has found that citrus flavanone hesperidin has antioxidant and anti-inflammatory effects (Haidari et al., 2015). Because inflammation plays a major role in chronic diseases, such as heart disease, the effect of hesperidin supplementation on inflammatory markers has become an area of research interest.

Hesperidin is known to treat a multiple disease, including lymphedema, haemorrhoids, venous stasis and varicose veins. It is similarly applied in therapy of cardiovascular and cerebrovascular diseases as well as in certain cases of abortion (Kar, 2003). Hesperidin is mainly applied during cancer operations. This means that it may be effective at any stage of cancer therapy. Although toleration of hesperidin

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DOI: https://doi.org/10.31024/ajpp.2024.10.4.3

supplements is quite high, there are possible side effects. The side effects relate primarily to gastrointestinal reactions.It involves diarrhoea and nausea (Milenkovic et al., 2011; Lee et al., 2004)

Typical hesperidin comes in the usage of dietary supplements such as vitamin C. But they often contain other bioflavonoids. Hesperidin is also presented in other forms such as supplements of the hesperidin complex, combinations with diosmin, different formulations with hesperidin and orange juice with pulp. Because of its forms, hesperidin is often referred to as an over-the counter drug. Rather, it is regarded as a supplement (Lee et al., 2004).

Objective of present study was to prepare hesperidin ointment and evaluated for burn wound healing in animals.

Material and methods

Preparation of hesperidin ointment

When waxy material and hand bodies are to be incorporated in soft oleaginous materials, fusion is the best technique. It consists of subdividing the waxy components and melting together all components over a water bath starting from one having the highest melting point. Remaining components are then added in order of their decreasing melting points till all the components are added up. Thus all components are not unduly exposed to higher temperatures. The mixture should be continuously stirred until congealed to ensure a homogenous preparation (Jain and Sharma, 1998). For the preparation of 30g ointment each fatty ingredient Hard Paraffin, Cetostearyl Alcohol and White Soft Paraffin were weighed and melted in China dish in decreasing order of melting point with continuous stirring. In other china dish Wool fat and hesperidin (2.5 and 5%w/w) was mixed and added in previously melted fatty substances with continuous stirring until cold.

Evaluation of prepared hesperidin ointment

Ointment prepared by fusion method and evaluated using different evaluation parameters such as pH, physical appearance, diffusibility, extrudibility using standard methods.

Burn wound healing effect

Animal grouping

Wistar albino rats (150-200g) of either sex were selected for the experiment. They were housed individually in well-ventilated, temperature controlled (26±2°C) animal room for seven days of period prior experiment. The animals were given the standard commercial pellet rodent diet (Hindustan Lever Pvt. Limited, Bangalore, India) and water ad libitum. The procedures were reviewed and approved by the Institutional Animal Ethics Committee.

For each group of animal, 6 animals were randomly selected and were divided into 4 groups. Animals in group-I received simple ointment base and served as control. Animals in group-II received topical application of 2.5 % w/w Hesperidin. The group-III received 5 % w/w Hesperidin. The group-IV received 5 % w/w Povidone Iodine Ointment USP (Zenith Drugs Pvt. Ltd, India) and served as reference group. The prepared formulations and reference ointment were applied twice daily to treat different groups of animal. Healing property was assessed using physical, biochemical parameters along with histopathological study.

Burn wound creation

Full thickness burn injury was induced in all rats according to the methods of Nakae and Inaba (Nakae and Inaba, 2000). In this method, rats were anesthetized by light ether. Hair on the back of each rat was removed with an electric clipper, and dorsal skin surfaces were exposed to 95^0C water for 10 seconds through a template designed to produce a third degree burn. The prepared formulations and reference ointment were applied daily up to complete healing. After day 20^{th}, the skin tissue samples were collected for biochemical estimation as well as histopathological study.

Wound Contraction and epithelialization time

The contraction of individual wound of control and treated animals were periodically measured using transparent graph sheet and rate of healing calculated and expressed as percentage contraction. The following formula was used to calculate percentage of wound contraction:

$$\text{Percent wound contraction} = \frac{healed\ area}{total\ area} \times 100$$

Wound contraction was measured in each two days interval

Hydroxyproline measurement

Small samples of tissues protein were hydrolyzed without preliminary purification by adding HCL to a final concentration of 6N. The samples were sealed in small pyrex text tubes and hydrolyzed for 3 hrs at 130 ^{0}C. The tubes were opened and the contents are decanted into a graduate cylinder or volumetric flask. Several drops of 0.02% methyl red indicator was added, followed by the theoretical amount of 2.5N NaOH required for neutralization. A final adjustment was made with dilute HCL and NaOH until the indicator turns slightly yellow corresponding to pH 6-7. The samples were prepared as above and 2.0-ml portions containing hydroxyproline was placed in test tubes. Hydroxyproline oxidation was initiated by adding 1 ml Chloramine T to each tube in a predetermined sequence. The tube contents are mixed

by shaking a few times and allowed to stand for 20 min at room temperature. The Chloramine T was then destroyed by adding 1ml of Perchloric acid to each tube in the same order as before. The contents are mixed and followed allowed to stands for 5 min. Finally 1 ml of p-dimethylaminobenzaldehyde solution was added and the mixture was shaken until no schlieren can be seen. The tubes were placed in 60 ^{0}C water bath for 20 min then cooled in tap water for 5 min. The developed color was stable for at least one hr. The absorbance of the solution was determined using UV visible spectrophotometer (Shimadzu, Japan) at 557 nm. The hydroxyproline content was determined directly from the standard curve (Woessner, 1961).

Protein estimation

The tissues were homogenized and precipitated with 5% Trichloroacetic acid [TCA] and the lipids removed by successive extraction with 0.1N potassium acetate in ethanol, ethanol and isopropyl ether. The acetate was added to neutralize the acid and prevent solution of some protein in the ethanol. The tissue lysate was treated with a mixture of sodium tartrate, copper sulphate and sodium carbonate. This was left to stand for 10 minutes and then treated with Folin-Ciocalteau reagent that resulted in a bluish color in 20-30 minutes. The absorbance was measured in UV (Shimadzu, Japan) Spectrophotometer at 650 nm (Lowry et al., 1951).

Enzymatic and non-enzymatic antioxidant assay

In dead space wound model, one part of granuloma tissue was used for antioxidant assay. Catalase was estimated following the breakdown of hydrogen peroxide according to the method of Beers and Sizer (1952). Superoxide dismutase (SOD) was assayed according to Misra and Fridovich (1972) based on the inhibition of epinephrine autoxidation by the enzyme. Reduced glutathione (GSH) content was determined in granuloma tissue by the method of Moron *et al*, 1979.

Histopathological study

Animals were anaesthetized before taking skin sample using diethyl ether. Wound tissue specimen from control, treated and reference group were collected and store in 10% formalin after that usual processing 6 µm thick sections were cut and stained with haematoxylin and eosin (McManus and Mowry, 1965). The histopathologic criteria were used with each animal for: epithelial proliferation, granulation tissue formation and organization, newly formed capillaries (identified by the presence of erythrocytes in their lumen) per site. Sections were qualitatively assessed under light microscope and were observed fibroblast proliferation, collagen maturation, angiogenesis and epithelialization.

Statistical analysis

The data were expressed as mean standard deviation (SD). The statistical significance of the difference in each parameter among the groups was evaluated using one-way analysis of variance (ANOVA) followed by the followed by the multiple comparison test of Tukey–Kramer tests. Criterion for statistically significant difference was chosen to be at $P<0.05$.

Results and discussion

Wound healing process consists of different phases such as granulation, collagenation, collagen maturation and scar maturation are some of the many phases, which are independent to each other. Hence in this study different wound models were used to assess the effect of herbal ointment on various phases.

Prepared hesperidin ointment (2.5 and 5%w/w) by using simple ointment base B.P. by fusion method and evaluated using different evaluation parameters such as pH, physical appearance, diffusibility, extrudibility. Rate of diffusion increased with increasing time. pH of the prepared formulation was found to be between 6-7 which is almost similar to the pH of the skin. Ointment was found to be stable even at elevated temperatures also (40^{0}C). Prepared ointment was found to be non irritant to the rabbit skin. Extrudibility was calculated to be 3.9 x 10^{-3} cm/g.

The prepared hesperidin ointment formulations were

Table 1: Effect of Hesperidin ointment (HO) formulations on percent wound contraction area of full thickness burn wound in rats

Groups	Post wounding days (Percent wound contraction)										Epithelialization
	2	4	6	8	10	12	14	16	18	20	period
Control (Simple ointment base)	6.25±0.05	10.34±0.42	13.75±0.62	19.20±0.28	27.54±1.08	33.46±1.65	42.18±2.85	52.84±2.68	61.75±3.14	70.29±3.75	24
HO (2.5% w/w)	9.62±0.04	17.34±0.37	25.20±0.42	30.58±1.34	41.36±1.85*	53.77±2.67*	67.42±3.01*	78.95±3.75*	87.44±3.86*	100	20
HO (5% w/w)	14.60±0.07*	29.47±1.08*	38.92±1.68*	51.62±2.07*	65.20±2.88*	71.63±3.14*	88.14±3.52*	93.27±3.75*	100	-	18
Reference Ointment	12.68±0.24*	28.88±1.05*	40.12±1.67*	52.38±2.55*	64.39±3.17*	73.20±3.25*	84.63±3.64*	91.24±3.88*	100	-	18

n = 6 albino rats per group, value represents Mean S.D. P< 0.05, when compared each treated group with control group; HO: Hesperidin ointment

evaluated for effect on full thickness burn wound model in rats. The marketed formulation, 5 % w/w Povidone Iodine Ointment USP (Zenith Drugs Pvt. Ltd, India) was used as a reference for comparison. The simple ointment base B.P. was used as a control for comparison.

Burn wound closure was measured on two days interval up to 20 days. The wound contraction form days 12 to 18, was found significantly ($P < 0.05$) faster in rats treated with hesperidin ointment and Reference ointment groups than the control group (receiving only simple ointment base).

We point out that burn wounds treated with ointment base alone did not achieve full-closure by day 20, while wounds treated with HO 5%w/w and Reference ointment groups was found complete healing in 18 days. In addition, treatment with 2.5 % w/w HO ointment treated groups of animals were found wound closure occurred in 20 days (Table 1).

The results of hydroxyproline content in the granulation tissue of the treated and control group of wounds are shown in table 2. The hydroxyproline content in animals treated with HO 5%w/w (54.87±1.75), HO 2.5% w/w (40.36±1.34) and reference ointment (52.68±2.16) was found significantly ($P< 0.05$) greater than control group of animals.

Over the 20-day treatment period, the protein content of wounds treatment groups with HO 5%w/w (72.33±2.85), and reference ointment was found significantly ($P< 0.05$) increased than control group of animals.

The content of antioxidants in skin tissues are given in table 3. Significant decrease in antioxidant level was observed in control group as well as in 2.5 % w/w HO and 2.5 % w/w HO ointment treated animals when compared with reference group. The antioxidant (SOD and GSH) level was increases in dose dependent manner.

Observation of histopathological photograph shows that control group doesn't show an acceptable wound healing activity. In case of reference and HO treatment group showed almost similar wound healing activity was achieved.

The skin lesion from control group of animals observed signs of a chronic active inflammation, either co-existing with ulceration or progressing as a sequel to ulceration. All skin section had acanthosis and dermal fibrosis, while others had some surface exudates, hyperkeratosis and other changes including epidermal inclusion cysts. The group treated with HO and Reference ointment observed that the wounds revealed formation of angioblasts and fibroblasts through out the tissue along with infiltration of neutrophils and microphages at few place on 18th days. Both group of treatment wound showed the formation of fibrous connective tissue and blood capillaries. The arrangement of blood capillaries was parallel to that of fibrous tissue at some place while it was perpendicular to that of fibrous tissue at other places. However, epidermal covering at few

Table 2: Effect of prepared hesperidin ointment formulations and reference ointment on hydroxyproline and protein content of tissues from full thickness burn wound in rats

Groups	Hydroxyproline content (mg/g tissue)	Protein content (mg/g tissue)
Control (Simple ointment base)	31.42±0.85	43.26±1.37
HO (2.5% w/w)	40.36±1.34	50.27±2.01
HO (5% w/w)	54.87±1.75*	72.33±2.85*
Reference Ointment	52.68±2.16*	70.24±2.67*

n = 6 albino rats per group, value represents Mean S.D. *P< 0.05, when compared each treated group with control group

Table 3: Effect of prepared hesperidin ointment formulations and reference ointment on enzymatic and non-enzymatic level of tissues from full thickness burn wound in rats

Animal groups	Enzymatic and non-enzymatic assay		
	SOD(µg/50mg tissue)	CAT(µmol/50 mg tissue)	GSH(µmol/50 mg tissue)
Control (Simple ointment base)	13.28±0.24	32.54±1.07	21.08±0.85
HO (2.5% w/w)	18.74±0.78	37.12±1.52	34.29±1.67
HO (5% w/w)	27.36±0.88*	39.25±1.77*	42.69±1.76*
Reference Ointment	26.54±0.96*	45.11±2.07*	43.22±1.88*

n = 6 albino rats per group, value represents Mean S.D. *P< 0.05, when compared each treated group with control group

places was seen on 20th days.

Conclusion

The results of present study showed that Hesperidin ointment possesses a definite healing action. This is demonstrated by a significant increase in rate of wound contraction and by enhanced epithelization. Significant increase was also observed in hydroxyproline content which was a reflection of increased collagen levels that was further supported by histopathological evidence. This indicated improved collagen maturation by increased cross-linking indicated higher protein content. An increase in the level of antioxidant enzymes was observed in burn wound model. These enzymes are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals.

Conflict of interest: None

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