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Determination of wound healing activity of Shark liver oil using the excision wound model in Wistar rats

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ABSTRACT

There are various promising preclinical models, such as in mice, wistar rats, rabbits and pigs, which can be utilized to initiate acute or chronic wounds. These can be persuaded by many distinctive techniques, with excision the most common. After determining a proper model for a study, investigators need to choose an appropriate and reproducible technique that will permit the monitoring of the wound improvement over time. In this study, the healing power of Shark Liver Oil Emulgel (SLO) in Wistar rats were analyzed by using the excision wound model. The shark liver oil was prepared as emulgel at a concentration of 5%,10% and 15%, respectively and both Standard drug (Povidone-Iodine Ointment USP 5% w/w-PI) and SLO is applied at a concentration of 1mg/mm^2; Topically. The parameters integrated for the assessment of the effects of SLO were Relative body weight changes, wound area contraction in mm^2, relative wound percentage and epithelialization time. Wound area contraction was measured on 1^{st}, 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th}, $18^{th}$and 21^{st} day. Wistar rats treated with SLO showed substantial variations in epithelialization period and improved wound contraction in the excision wound treatment as compared to disease control. The biochemical biomarkers like SOD (Superoxide dismutases), GSH (Reduced Glutathione)and LPO (Lipid peroxidase) in the treated group have shown a significant change in the improvement of wound healing. Histopathological studies and microscopic observations specify that the topical use of Shark liver oil Emulgel extensively improved wound contraction, collagenation and epithelialization with well-organized dermis devoid of inflammatory cells in contrast to disease control.

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INTRODUCTION

Wounds are life-related incidents that may be due to physical trauma, chemical accidents or microbial infections. Wound cure has three separate phases: infection, development, and restructuring (Witte and Barbul, 1997; Singer and Clark, 1999). Drug discovery that stimulates the mechanism of wound healing is a domain of clinical and biomedical sciences. Various medicines from marine sources and plant sources have been documented to improve the cure of various forms of a wound (Adedapo *et al.*, 2008). The use of traditional medicine has been a critical component of health care in mod-

ern days due to a combination of traditional use over years and current accelerated science studies. Conventional treatments such as antimicrobial drugs, anti-inflammatory drugs and antibiotics have proven their effectiveness in resistance development in many infectious organism-related diseases (Gold and Moellering, 1996). Thus, the use of natural preparations in day to day life are more effective than traditional drugs and can be used for more extended periods, provided they posses non-toxic properties (Argolo, 2004). To establish the theoretical basis for the stated wound healing behaviour of natural agents as a single agent or in a type of formulation, the above criteria are taken into account, and further detailed research has been carried out. The research formulation of SLO comprises normal and pure shark liver oil (Bordier *et al.*, 1996). Formulations are formulated in the form of emulgel(SLO-Shark liver oil Emulgel) in a concentration of 5%,10% and 15%, based on the traditional concepts for its antioxidant properties of shark liver oil (Joelssun *et al.*, 1997).

SLO is also recommended in patients diagnosed with a topic dermatitis caused by bacterial and fungal infections (Nowicki and Barańska-Rybak, 2007). SLO has been used in the therapy of wound healing, and for treating irritations of the respiratory plus alimentary tracts and lymphadenopathy (Palmieri *et al.*, 2014). A systematic analysis of the fundamental science of healing is given in shark liver oil supplements in which it is also known as an incredible healer. Shark liver oil is efficient in the treatment of healings (Debouzy *et al.*, 2008)**.** In the present study, we have performed this research to test and measure the effectiveness of these formulations with the excision wound paradigm in laboratory Wistar rats.

MATERIALS AND METHODS

Materials

Both analytical quality chemicals such as sodium hydroxide ketamine, sulphuric acid Methylparaben, Carbapol, Tween 80, Span 20 and other analytical grade reagents M/s SK APPLIANCES, Ambala Cantt, Punjab, with voucher number SKA/2019-20/10025 were purchased accordingly. Mr Nissar FM, Coastal Exports Corporation, Mangalore, India, procured Shark Liver Oil as the gift sample.

Methods

Shark liver oil is a complex mixture of triglycerides and alkylglycerols (also known as ether lipids) (Deprez *et al.*, 1990; Pugliese *et al.*, 1998) in which high amounts of squalene are found in the completely refined shark liver oil (Tsujimoto, 1920). The shark liver oil is extracted from the fresh liver of numerous Squalidae (e.g. Squalus Blainville) and Centrophoridae (e.g. Centrophorus zeehaani) family The livers are alienated from the sharks, cleaned and are unbound from fatty matter and adhering tissues. The untainted livers are accumulated and heated in a boiling container at a temperature, not above 80 ^{0}C. The extracted oil is then passed on to dehydration process to flush out water and centrifuged to get clear oil. Shark liver oil obtained is 100% pure, free from artificial colouring, preservatives and additives (Bordier *et al.*, 1996; Bañón *et al.*, 2008).

Preparation of Emulgel

Shark liver oil Emulgel was made by the method reported by Mohamed (2004) with slight modification. The Gel in formulations was prepared by dispersing Carbopol 934 in purified water with steady stirring at a moderate rate and Carbopol 940 in purified water with constant stirring at a moderate speed then the pH are regulated to 6 to 6.5 using Triethanolamine (TEA). The oil phase of the emulsion was made by dissolving Span 20 in light liquid paraffin whereas the aqueous phase was prepared by dissolving Tween 20 in purified water. Methyl and Propylparaben were melted in propylene glycol whereas SLO was dissolved in ethanol and both solutions were blended with the aqueous phase. Mutually the oily and aqueous phases were independently heated to 70° to 80°C; then the oily phase was combined with the aqueous phase with constant stirring until cooled to room temperature. Glutaraldehyde is added during the mixing of Gel and emulsion in ratio 1:1 to obtain the emulgel (Mohamed, 2004).

Animal

Male Wistar Rats (150 to 250 grams) were obtained from Shri Mahaveer Enterprises, Hyderabad from animals of healthy adults. Animals in study polypropylene cages were lodged in five groups. They were kept in 23±2ºC temperature with relative humidity between 30to70% and 12:12 dark light periods. Pathogenicity was checked in animals by acclimated for two weeks and held free. The regular plate chow was readily available to livestock. Purified water was given to animals during the research procedure. The Institutional Animal Ethical Committee (IAEC)of the Pretox Research Centre, Surat, Gujarat has gained the ethical clearance in animal experiments with protocol number PRC/AFC/2019/310. Standard operating procedures and CPCSEA guidelines have been followed for animal experimentation. The animals have been classified into five different groups, each with six

adult wistar rats.

Experimental Design

The following groups were allocated to the experiment of the excision wound model.

Group -1 Disease Control (DC)-did not receive any treatment.

Group -2 (Povidone-Iodine Ointment USP 5% w/w-PI) or Standard Drug (STD)

Group -3 Shark Liver oil Emulgel 5% (SLO 5%)

Group -4 Shark Liver oil Emulgel 10% (SLO 10%)

Group -5 Shark Liver oil Emulgel 15% (SLO 15%)

Excision Wound Model

Excision wound was created with slight modification as per the method described. Five groups of animals each containing six rats were shaved on the dorsum portion using depilatory cream (Reckitt Benckiser, Inc., UK) and anaesthetized using ketamine hydrochloride (50 mg/kg, i.p., bodyweight). An impression was made on the shaved dorsal region and area of the wound to be created was marked. A full-thickness excision wound with a circular area of 314 mm^2 was created along the marking using toothed forceps, a surgical blade, and pointed scissors. Rats were left undressed to the open environment. The formulated SLO and standard drug were applied once daily from the day of the operation until the complete healing. In this model, wound contraction and epithelialization period were evaluated. Wound contraction was measured as per cent contraction every 3^{rd} day after wound formation. At the end of the study, all the rats were anaesthetized, and from the healed wounds, specimen samples of tissue were collected from each rat, leaving a 5 mm margin of normal skin around the edges of the healed wound. Specimen tissues were stored in 10% formalin solution and used for histopathological and biochemical studies. The wound dressing was performed daily, and the study formulations were applied daily to the wound surface for the 21 days. The wound area contraction was measured on 1^{st},3^{rd}, 6^{th}, 9^{th}, 12^{th},15^{th},18^{th}, 21^{st}day. As the endpoint of full epithelialization, dropping scar leaving no raw wound behind was taken and the days needed for this were taken as the epithelialization period (Mukherjee *et al.*, 2000).

Histopathological examinations

Healing tissue samples have been collected from all five animal groups and have been assessed for histological analysis. The samples were fixed in formalin and mounted on slides labelled with Hematoxylin and Eosin and then studied under an optical light microscope. Inflammatory lesions, fibroblast proliferation, neovascularisation, epidermal hyperplasia, and Ulceration were the reporting parameters under the histopathology study.

Statistical Analysis

Outcomes are expressed as mean ± S.E.M. The discrepancies between the study groups were contrasted with Dunnett's and Tukey's Multiple Comparisons Test by one and two-way analysis of variance (ANOVA) (DC vs treatment and STD vs treatment). They were found statistically significant at (*p<0.05, **p<0.01) (Armitage *et al.*, 2002).

RESULTS

It is inferred from the present analysis that there is a substantial decrease in epithelization after using Shark Liver Oil Emulgel (5%,10% and 15%) compared to the regular standard drug. Therefore, comparative research has shown that formulations of SLO possess significant wound healing effect.

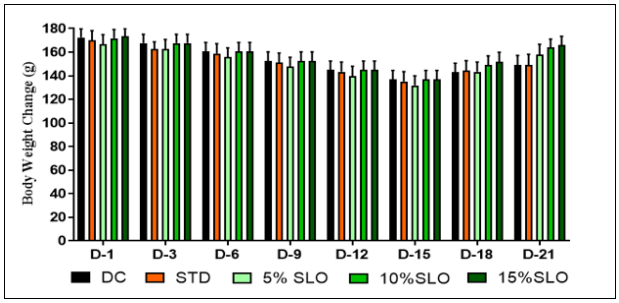

Figure 1: Bodyweight changes

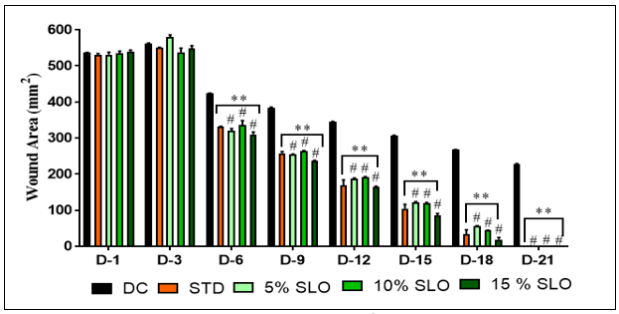

Figure 2: Wound area in mm^2

In $, the results have shown an insignificant increase in body weight from 15 days post wound.

In Figure 2, there is a significant increase in wound healing activity of Shark liver oil Emulgel treated groups in comparison to the disease control group. All values were represented as Mean±SEM, *n*=6, Data was analyzed by Two-way ANOVA, followed by Tukey's Multiple Comparisons Test. *p<0.05, **p<0.01 represent significantly different as compared to DC and #p>0.05 represents non-significant in comparison to Povidone Iodine.

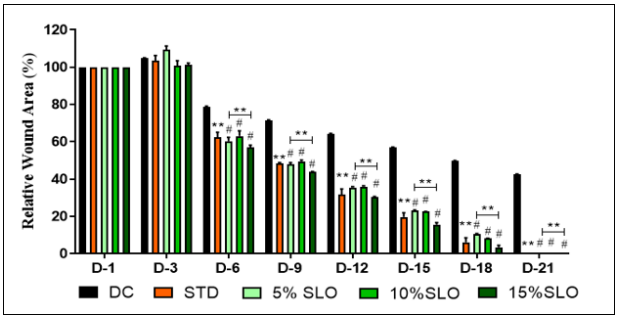

Figure 3: Relative wound area (% Percentage)

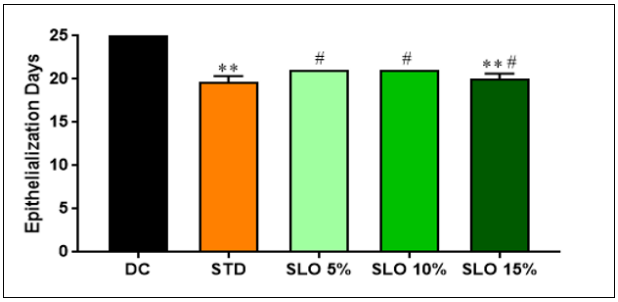

Figure 4: Epithelialization period

In Figure 3, it has shown a significant reduction in relative wound area in % Percentage of different groups for 21 days. The area of the wound was measured on the 1, 3, 6, 9, 12, 15, 18 and 21 days of post wound surgery in all groups. All values were represented as Mean±SEM, *n*=6, Data was analyzed by Two-way ANOVA, followed by Tukey's Multiple Comparisons Test. *p<0.05, **p<0.01 represent significantly different as compared to DC and #p>0.05 represents non-significant in comparison to Povidone Iodine.

In Figure 4, a very high rate of closure of wound and epithelialization was observed post 19 days in treated groups. Shark liver oil Emulgel and Standard groups have shown significant wound healing activity(*p<0.05, **p<0.01) and gradual closure of the wound by 20th day of post-surgery and by 25 days in control groups. All values were represented as Mean ±SEM, *n*=6, Data was analysed by One-way ANOVA, followed by Dunnett's Multiple Comparisons Test. *p<0.05, **p<0.01 represent significantly different as compared to DC and #p>0.05 representnon-significant in comparison to Povidone Iodine.

In Figure 5(A-C), Shark Liver Oil Emulgel has shown significant improvement in the wound healing process, and the antioxidant property has resulted in decreasing SOD, LPO and increasing GSH activity in all treated groups. This proves significant wound healing activity of Shark liver Oil Emulgel. All values were represented as Mean ±SEM, *n*=6, Data was analyzed by One-way ANOVA, followed by Dunnett's Multiple Comparisons Test. *p<0.05, **p<0.01 represent significantly different as compared to DC and represents #p>0.05 non-significant in comparison to Povidone Iodine.

In Figure 6(A-E), in the present investigation, from histopathological findings and Microscopic observation shows that topically applied *Shark liver oil Emulgel* has significantly enhanced wound contraction, collagenation and epithelialization with well-organized dermis devoid of inflammatory cells as compared to disease control. Histological examination of the haematoxylin stained tissue of the rat wounds treated with test drug and Standard has shown superior healing in lesion numbers as shown in histograms. All values were represented as Mean ±SEM, *n*=6, Data was analyzed by One-way ANOVA, followed by Dunnett's Multiple Comparisons Test. *p<0.05, **p<0.01 represent significantly different as compared to DC and represents #p>0.05 non-significant in comparison to Povidone Iodine.

DISCUSSION

The aim of this research was to explore the effects of the shark liver oil emulgel in experimental rats on four key parameters, Relative Body weight changes, wound area contraction, collagen formation and period of epithelialization. Numerous growth factors such as growth hormone(GH) resembling Granulocyte-macrophage colony-stimulating factor(GM-CSF)growth factors and Insulin-like growth factor reverse the high rate of catabolism after wound injury by influencing metabolism and alters body weight (Jiang *et al.*, 1989; Kagan *et al.*, 1995). Tissue destruction after thermal injury were the outcome of an energy insufficiency which produces hypermetabolism initiated by decreased food intake instantly after injury. The injured animals in this test illustrated large growth retardation, were able to recuperate their body composition to that of an uninjured animal of the same weight (Drury, 1976). It is observed that there is a non-significant increase in relative body weight post 15 days of excision (Figure 1). The wound healing mechanism primarily relies on the controlled collagenic production of new collagen deposition and its consequent ripeness (Puratchikody *et al.*, 2006). Collagen is found in abundant quantity in our body. In wound healing, it plays an important role by attracting fibroblast and encourages the deposition of new collagen to the wound bed, which stimulates new tissue growth and promotes angiogenesis and re-epithelialization (Baum and Arpey, 2006). Wound Contraction is a healing mechanism accomplished by two vital proteins actins and

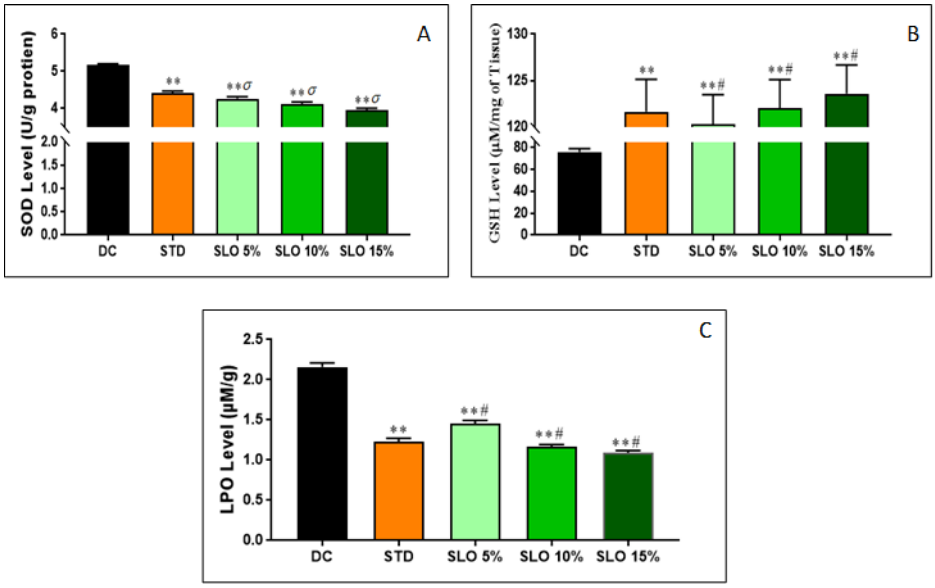

Figure 5: Antioxidant activity

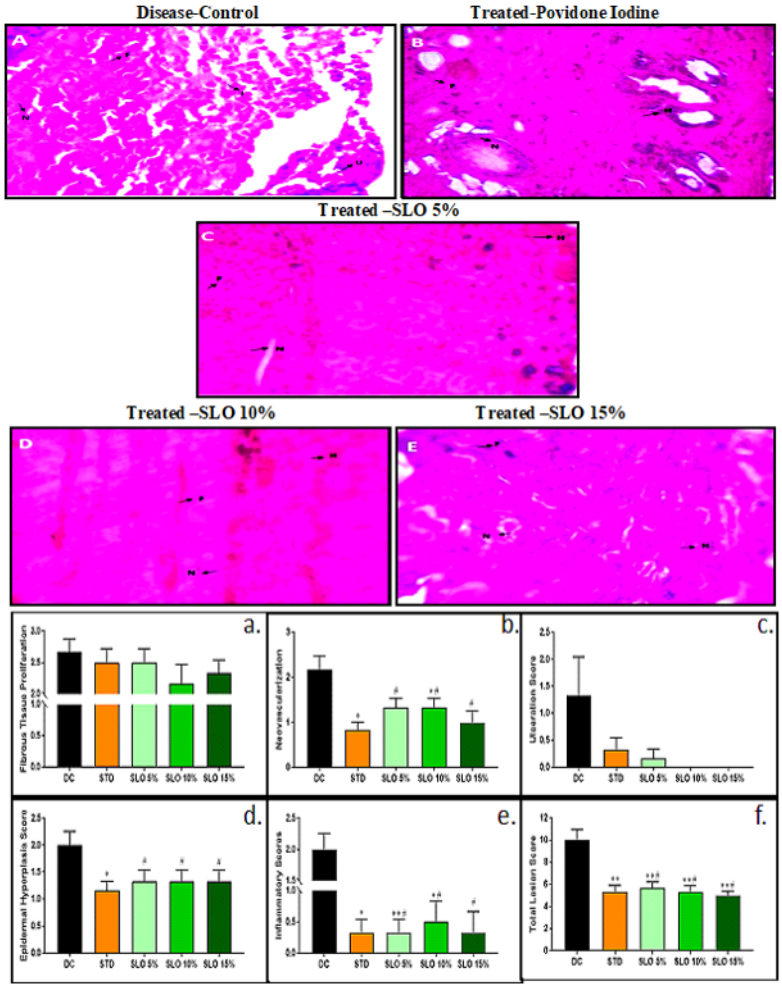

Figure 6: Histopathology and Microscopic observation

myosin. It characterized by the movement of fibroblasts in granulation tissue collagen and pulling the forces of granulation tissue myofibroblasts on the skin edges (Gabbiani, 2003) (Figure 2, Figure 3). Epithelialization is characterized by the migration of epithelial cells in an upward direction and repairing the wounded area, which mainly occurs at the proliferative phase of wound healing (Figure 4). The study shows that the topically applied shark liver oil emulgel (5%, 10% and 15%) has supported collagenation, wound contraction and epithelialization and has proven imperative in comparison to other fixed oil (*p<0.05, **p<0.01) (Modarresi *et al.*, 2019) GSH (reduced glutathione levels), LPO (Lipid peroxidase) and SOD (Superoxide Dismutase) are the big biochemical biomarkers for tissue damages such as cut wound and skin ulcers (Figure 5).

Lipid peroxidation (LPO) causes the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism (Yin *et al.*, 2011).

Superoxide dismutase (SOD) an endogenous antioxidant, are a group of enzymes that catalyze the dismutation of superoxide radicals (O2−) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) by providing cellular defence against ROS (reactive oxygen species) (Fridovich, 1995). Reduced glutathione (GSH) acts as an effective antioxidant and protects the cellular components from oxidative damage caused by ROS(Reactive Oxygen Species) (Pompella *et al.*, 2003). In our study as an endpoint parameter analysis reduced level of LPO and SOD and increased level of GSH showed SLO formulation's antioxidant efficacy against skin tissue damage.

The preliminary qualitative screening for shark liver oil discovered squalene and alkylglycerols to play a significant role in promoting wound healing via multiple mechanisms (Huang *et al.*, 2009; Lewkowicz *et al.*, 2006). In this analysis, a high concentration of active ingredients may be the cause for having curing ability of the SLO which are responsible for its antioxidant, anti-inflammatory, antifungal and wound healing effects in contrast with other fish oils (Nowicki and Barańska-Rybak, 2007; Tsujimoto, 1920; Villani *et al.*, 2013).

Povidone-iodine is a well accounted antimicrobial medication as routine wound care that is used to avoid second-hand disease infections (Fleischer and Reimer, 1997). In comparison to Standard as has been described, the Shark liver oil emulgel also possess the significant ability for curing of wounds.

Skin discomfort tests found that Emulgel formulations for Shark liver oil do not display significant kinds of discomfort in a topical application on the skin (Gfeller *et al.*, 1985). This indicates that the formulation consists of some compounds that are chemically non-irritating and that they may allow shark liver oil Emulgel to repair the damage. In Histopathology, we scientifically examine the alterations in the affected tissues under the microscope even though the fact that its an ancient method but still being practice in medical sciences (Subbarayappa, 2001).

Histopathology study is characterized by Fibroblast proliferation, Neovascularization, Epidermal Hyperplasia, Inflammatory score and Ulcer formation. Fibroblast proliferation comprises of Fibroblasts which are essential in the process of tissue repair. They produce structural proteins, adhesive proteins and space-filling ground substance imperative in building extracellular matrix(ECM) of connective tissue (Mutsaers *et al.*, 1997). Neovascularisation is also recognized as new blood vessel formation, is very eminent in successful tissue restoration during wound repair. The new blood vessels formed assists in supplying oxygen and nutrients from blood to damaged tissue (Kalka *et al.*, 2000). Epidermal hyperplasia is an important response of connective tissue in which the proliferating fibroblasts and blood vessels help in wound repair (Raj *et al.*, 2006). An inflammatory response is illustrated by damaged cells, pathogens and elimination of Bacteria in which growth factors, white blood cells(WBC), enzymes and nutrients instigate redness, pain and swelling (Martin and Leibovich, 2005).

Ulcers due to arterial insufficiency often commence as minor traumatic wounds that fail to heal. The primary strategy to nurture arterial insufficiency ulcers is to reinstate proper blood flow to the tissue to enhance appropriate wound healing (Ortonne and Clévy, 1994). Histopathology and Histopathological scoring method has verified the effectiveness of SLO administration in the development of the adverse effects on Excision wound healing and encompasses semi-quantitative scoring of the inflammatory phase, the amount of granulation tissue, level of re-epithelialization, amount of collagen deposition, level of Fibroblast proliferation, Neovascularization, Hyperplasia and Ulceration (Abramov *et al.*, 2007). The total healing score indicated that lower scores verified inferior wound healing in Control groups in contrast to significant healing grade in SLO treated groups (Figure 6).

The findings of this study, therefore suggest that

Shark liver oil emulgel's topical application has improved wound care in laboratory rats using an excision wound model.

CONCLUSION

In this study, the results have revealed that the Shark liver oil emulgel has active ingredients which promote the natural treatment of wounds and can be used as a wound cure agent effectively. Shark liver oil emulgel increases the solidity of the wound and the epithelialization rate and longevity of the collagen throughout the surface of the wound. The concentration-related healing effect in laboratory rats was seen in all three formulations. Further detail study is requisite for investigation of wound healing properties and mechanism of action.

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Conflict of Interest

All authors have thoroughly read the whole manuscript and declared no conflict of interest.

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