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COMPARATIVE STUDY OF FORMULATED WOUND HEALING OINTMENTS WITH THE MARKETED PRODUCT BY *IN-VIVO* METHOD

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ABSTRACT: A wound is a breakthrough of skin; it disrupts the normal functioning of skin. So, the wound healing process is important to repair the skin. It restores the normal function and structure of the skin. There are several phases of the wound healing process: inflammation, re-epithelization, vascularization, and granulation tissue formation and maturation. The wound healing process can be studied using *in-vitro* and *in-vivo* methods. There are many *in-vivo* methods to study the wound healing process. *In-vivo* method uses laboratory animals for the study. Animals used are mice, rats, rabbits, and pigs. Recently, Zebrafish have been used as a model to study the wound healing process. Zebrafish are used because it has similar wound closure properties as mammalian embryos. The wound healing can be studied by amputating the caudal fin of fish and recording the regeneration of the fin on days 5th, 7th and 9th post-amputation. This study compares various wound healing agents with the market product (neomycin cream). The various wound healing agents used in this study are pomegranate peel powder, Aloe vera powder, potash alum powder, lavender oil, and sesame oil. The wound-healing effects of these agents are studied using Zebrafish as an *in-vivo* wound-healing model.

INTRODUCTION: A wound is a breakthrough of the skin; it can disrupt the anatomical and functional continuity of the skin. It can be caused by heat, radiation, microbial, chemical, *etc*. Wounds can be classified based on location, etiology, presenting symptoms or type of injury, wound depth, and tissue loss or clinical appearance [1]. The structure of the skin is restored by a process called wound healing. Wound healing is a process to respond to tissue damage integrity of damaged skin tissue. Wound healing is a series of processes carried out in an orderly manner. It is a dynamic process that takes place by regeneration or repair of broken tissues; after the injury, collagen is necessary to repair the defect and restore the skin's structure and function. These processes depend on mediators like extracellular matrix molecules, platelets, inflammatory cells, growth factors, cytokines, and chemokines. Commonly, the process consists of three overlapping phases: inflammatory, proliferation, and remodeling phase [2].

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The progression of all these events leads to the successful completion of wound healing and restoration of the disrupted structure and function of the skin. Acute or normal wound healing processes occur in an orderly manner; in a healthy individual, the wound healing usually occurs in 7 - 10 days. Chronic wound healing refers to a disruption of the normal healing pathway, which can be caused due to underlying diseases, infections, medications, and old age. Chronic wound healing may take months to years to heal completely. Various wound care products are developed to stimulate the healing process, reduce scar formation, and improve the skin's properties. Many efforts have been made to explore new agents to enhance the healing time of the wound. Natural products with medicinal properties can facilitate the wound-healing process.

Various experimental wound-healing models have been developed to understand the wound-healing process. The models are of two types: *in-vitro* and *in-vivo* (animal) [3]. Various in-vitro methods include scratch cell assay, cell culture, and skin plant culture. *In-vivo* models are preferred because they give an idea about the wound healing process and give great results. In these models, the laboratory animals are wounded, and the time taken for wound closure is recorded. These ideal models represent certain aspects of human physiology. For the experiment, various species are used, including rats, mice, rabbits, and pig. Recently, Zebrafish is also used as a model for the wound healing process [4]. *In-vivo* models give a realistic representation of the wound-healing process. For *the in-vivo* study, we need ethical clearance from the board. Animals commonly used as models in the *in-vivo* study are rats and mice [5]. There are some differences between human skin and rodent skin structure, so the differences are kept in mind while designing the test. Rabbits are also used as models (rabbit ear excisional wound model). Pig skin is structurally and functionally similar to human skin [6].

Various models used for the evaluation of the wound healing process are:

- Incisional wound.
- Excisional wound.
- Burn wound

Incisional Wound: This method is used to investigate surgical incision materials. Animals used to evaluate wound healing are mice, rats, and rabbits. It is a fast wound closure and is dominated by collagen deposition. There are three types of incision wounds, primary closure and secondary closure and tertiary closure. The primary closure is an excellent model for the biochemical analysis of wounds. Secondary closure is used to investigate the scarring. They are involved in an experimental animal model to investigate the healing process, such as incisional injury in mice, to study tensile strength. Tertiary closures are delayed in the primary intention. Delay type of closure, closing the wounds and skin tissues [7].

Excisional Wound: Excisional wounds are created by surgical removal of skin layers, like the dermis, epidermis, and subcutaneous. It resembles the acute wound, so it is a commonly used model. Investigation of hemorrhage, inflammation, granulation tissue formation, re-epithelialization, angiogenesis, and remodeling can be done. The wound healing rate is evaluated by measuring the size of the wound. Animals used are mice, rats, rabbits, pigs and different types of wounds can be created.

Types:

Euthanized: The experiment wound healing model, developed over decades in an attempt to understand the tissue repair process and test new treatment protocols. In this model most commonly used animals like mice and rats.

Locally Anesthetized: In this experiment, the ability to create a large number of wounds and a relatively short time for evaluation. A biofilm is pathophysiological and is essential to develop wound care principles. Ex. Rabbit ear model [8].

Burn Wounds: Burn wounds are used for measuring the re-epithelialization depending on the depth of the burn. Burn wound healing models can also be generated by the scalding in the skin or thermal damage [9].

Methods Used to Determine Wound Healing Process:

- Wound healing rate.
- Wound analysis by image.
- Image analyses by software.
- Biophysical assessment of wound healing
- Histopathological analysis.
- Immunological methods.
- Biochemical methods.
- Macrophage polarization

Advantages of *In-vivo* Studies:

- Side effects can be easily viewed.
- It gives accurate results in a complex model.
- It is a clinically relevant method.
- Best way to enhance clinical performance.

Disadvantages of *In-vivo* Studies:

- *In-vivo* method is harder to control the variable.
- *In-vivo* experiments are expensive.

Zebrafish as a Model for Wound Healing Process: In recent years, the use of Zebrafish as a model for various *in-vivo* studies of human development and disease [10]. The principles used for wound closure are similar in the epidermis of Zebrafish and mammalian embryos [11].

In this paper, we studied the wound-healing effect of various wound-healing agents and compared it with a marketed product using a zebrafish as a model. The Zebrafish became an important and valuable model for studying the inflammation and wound repair process [12]. For the study, the caudal fins of the Zebrafish are amputated with a sterile needle. Then various formulations containing different wound healing agents are applied on the amputated caudal fin of the fish and allowed to recover, then transferred to the tank [13].

The growth of the caudal fin is measured on 5th, 7th and 9th day post amputation [14]. The growth is measured dorsal and ventral sides with the help of software N.I.H. image 1.62 software [15]. After applying the different wound healing ointments, the fin regeneration in different fish is compared with the standard (marketed drug). This study gives detail about the best wound healing agent.

MATERIALS AND METHOD:

Materials: Pomegranate peel, Aloe vera, Potash alum powders, Sesame oil, and Lavender oil were purchased on online websites Flipkart and Amazon. And all other chemicals were purchased from Merck Ltd. India.

Methods: The formulation of the ointment base is given in **Table 1**. The formulation of various wound healing ointments is shown in **Table 2.**

Ointment Preparation:

(a) All the powders were taken and then passed through a sieve number 120. The powder should be very fine to reduce the problem during binding.

(b) Initially, the ointment base was prepared by weighing accurately grated hard paraffin placed in an evaporating dish on a water bath. After melting hard paraffin, remaining wool fat, cetostearyl alcohol, and soft white paraffin are added and stirred gently to aid melting and mixing homogeneously, followed by cooling of the ointment base.

(c) Ointment was prepared by mixing accurately weighed fine powders with glycerine used as a levigating agent, then add ointment base by levigation method to prepare a smooth paste with 2 or 3 times its weight of base. Gradually incorporating more base until to form homogenous ointment. Finally transferred in a suitable container.

(d) Ointment was prepared by mixing accurately weighed oil which is previously heated, then added to the ointment base (mixture A) and the powder is mixed with glycerine (used as a levigating agent) and added to the above mixture A to prepare a smooth paste with 2 or 3 times its weight of base. Gradually incorporating more base until to form homogenous ointment. Finally transferred in a suitable container.

TABLE 1: FORMULATION OF OINTMENT BASE

Ingredients	Quantity to be taken
Wool fat	0.5gm
Cetostearyl alcohol	0.5gm
Hard paraffin	0.5gm
White soft paraffin	8.5gm

TABLE 2: FORMULATION OF OINTMENTS

Ingredients	F1	F2	F3	F4
Pomegranate peel powder	1 gm	-	-	0.35 gm
Lavender oil	-	1 gm	-	0.35 gm
Sesame oil	-	-	1 gm	0.35 gm
Aloe vera powder	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Potash alum powder	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Ointment base	8 gm	8 gm	8 gm	8 gm

Evaluation:

***In-vivo* Study:** The wound healing potential of the ointment is studied in Adult zebrafish. The fishes are wild caught in the bred. The source of fish is Whizbang Bioresearch Pvt. Ltd. Age of the fish is 5-6 months. Both the sex of fish are used for the study purpose.

Procedure:

Study Design: Fish was netted from their home tank and acclimatized in the holding tank for a week of time. Then, Zebrafish have anesthetized in 0.016% Tricaine and prepared for amputation [16]. The caudal fin of all study fishes was amputated in a straight line using a sterile scalpel to the proximal branch point of the dermal rays while viewing the fin through a dissecting microscope [17].

TABLE 3: FISH WERE DIVIDED INTO SIX GROUPS IN THE FOLLOWING MANNER

Group	Treatment/concentration	Total number of fishes
Group I	Amputated -control	10
Group II	Test item- F1	10
Group III	Test item- F2	10
Group IV	Test item - F3	10
Group V	Test item -F4	10
Group VI	Standard- Reference drug (Neomycin cream)	10

Following amputation, Group I to Group V fishes were treated with control, F1, F2, F3, and F4, respectively. Group VI was treated with a standard reference drug (Neomycin), which was carefully applied onto the transected fin with gloved hands under aseptic conditions [18]. Following treatment, fish were allowed to recover and transferred to the holding tanks of their respective groupings, as shown in **Table 3**.

The test item and standard drug were applied to the fish for 3 consecutive days post-amputation. Fish were maintained in the holding tank for 9 days post-amputation. Water was changed every day.

Physico-chemical Parameters of Exposure Medium: Water parameters in holding tanks were analyzed and maintained at optimum hardness, pH, Dissolved oxygen (D.O.), conductivity, and Temperature all over the experiment in controls and all the test groups.

Observation: The fish were again anesthetized, and images of the regenerating fins under a dissecting microscope equipped with a camera at IX magnification were taken at 5, 7, 9th day post-amputation to measure the area of regeneration [19]. The regenerative area was traced using N.I.H. Image 1.62 software [20].

RESULTS: After the amputation of the caudal fin, the regeneration of the caudal fin was measured. The area of regeneration of the amputated caudal fin was measured in mm. The measurement is shown in **Table 4.**

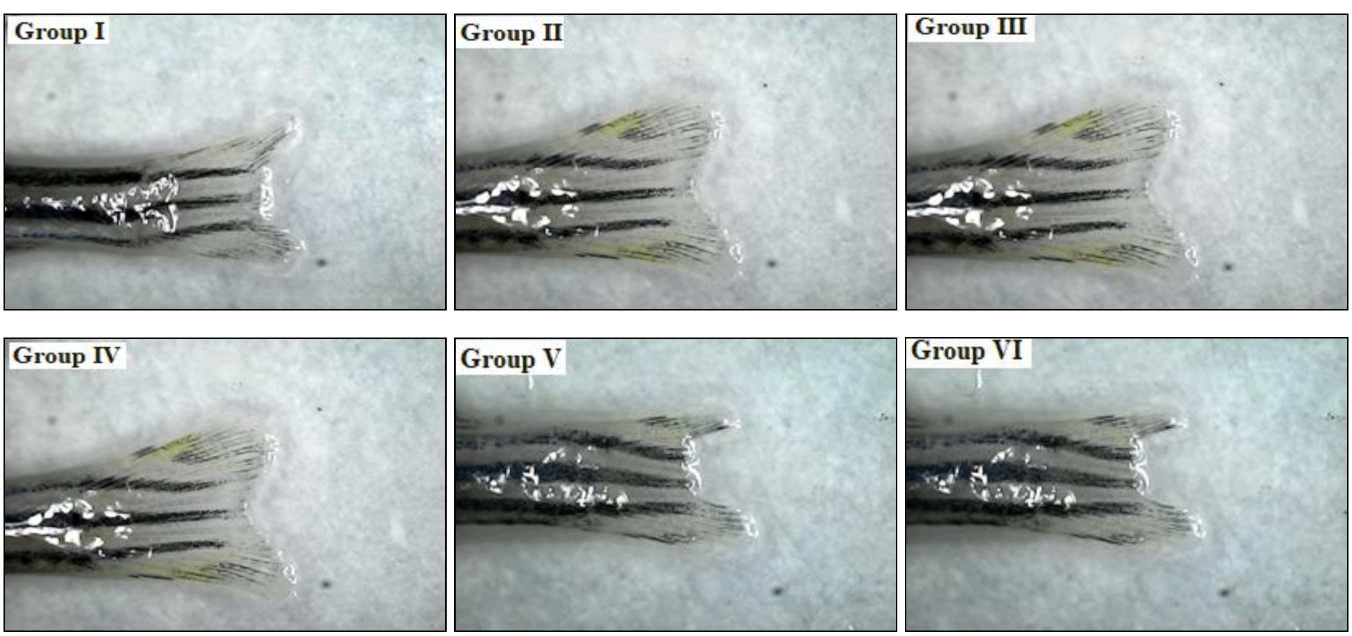

FIG. 1: REGENERATION OF AMPUTATED CAUDAL FIN ON 5TH DAY POST AMPUTATION

TABLE 4: AREA OF REGENERATION OF AMPUTATED CAUDAL FIN (MM)

		5dpa	7dpa	9dpa
Group I	Dorsal	1.340 ± 0.033	1.474±0.008	1.487±0.007
	Ventral	1.353±0.30	1,470±0.007	1.492±0.006
Group II	Dorsal	1.430±0.005	1.670±0.008	1.840±0.007
	Ventral	1.415±0.036	1.674±0.005	1.837±0.006
Group III	Dorsal	1.531±0.007	1.883±0.010	2.006±0.006
	Ventral	1.528±0.006	1.889±0.003	2.000±0.004
Group IV	Dorsal	1.500±0.009	1.731±0.005	1.889±0.004
	Ventral	1.505±0.009	1.728±0.005	1.892±0.005
Group V	Dorsal	1.400±0.004	1.580±0.005	1.709±0.006
	Ventral	1.405±0.005	1.585±0.008	1.702±0.008
Group VI	Dorsal	1.452±0.053	1.497±0.008	1.506±0.010
	Ventral	1.405±0.000	1.502±0.007	1.511±0.004

Note: dpa- day post amputation.

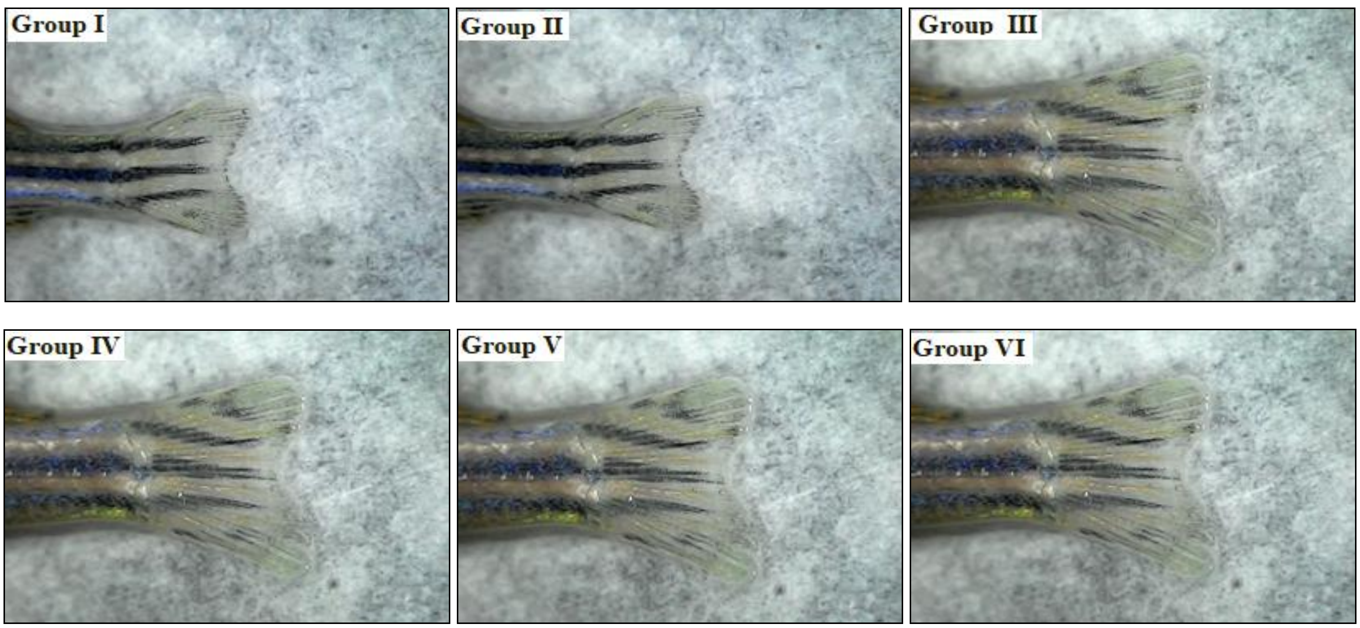

FIG. 2: REGENERATION OF AMPUTATED CAUDAL FIN ON 7TH DAY POST AMPUTATION

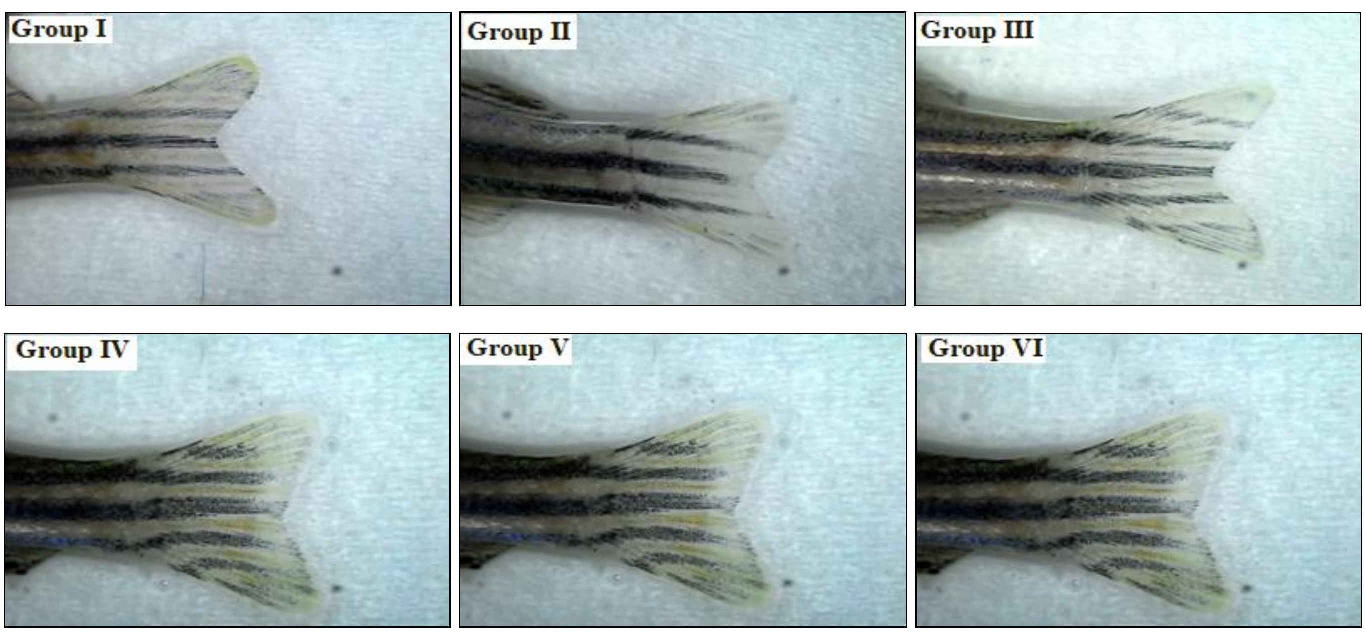

FIG. 3: REGENERATION OF AMPUTATED CAUDAL FIN ON 9TH DAY POST AMPUTATION

DISCUSSION: The result shows that the test item, ointment formulations F1, F2, F3, F4 exhibits a wound healing potential against caudal fin amputation in the adult zebrafish model under the present study condition. Significant variation was observed in the area of regeneration of Group II, III, IV and V when compared to Group I and Group VI. Test items (F1, F2, F3, and F4) exhibited enhanced wound healing effects in comparison with the standard reference drug (neomycin). Group III (F2) shows more wound-healing properties than all other Groups. So, the F2 formulation is more effective than the standard and all other wound healing agents used.

CONCLUSION: The ointment formulated with several wound healing agents are more effective in healing wounds. In the present study, we evaluated the properties of various wound-healing agents using Zebrafish as a model. F2 formulation was found to have a more wound-healing effect than all other formulations (F1, F3, F4). The formulations are compared with the marketed product (Neomycin), and the results are found to be more effective by *in-vivo* study.

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