

Evaluation of “Calendula 30” on the wound healing properties and related gene expression in Red Tilapia fish *Oreochromis sp.*

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Abstract

The present study investigated cut wound healing in the Red Tilapia fish *Oreochromis sp* using superficial application of the homeopathic medicine Calendula gel. Wound healing was assessed by examining microbial load, hematological parameters, histopathological changes, and the expression of selected wound healing-related genes, namely tumor necrosis factor- α (TNF- α) and transforming growth factor (TGF), at 0, 5, and 10 days post-wounding (dpw) in both control and treatment groups. The results of hematological analysis indicated that Calendula gel did not adversely affect immune-related blood parameters. Further, Fish treated with Calendula gel exhibited enhanced wound regeneration and early hyperpigmentation by 5 dpw, indicating accelerated tissue repair. Wound healing responses following treatment with Calendula gel and povidone-iodine were compared with untreated controls, which exhibited a relatively slower healing rate at 1th, 5th and 10th days. Histological examination revealed fragmented and disorganized microridges during the early stages of healing, suggesting their possible role in epithelial cell stretching and migration during wound re-epithelialization. The acute inflammatory response was substantially resolved by the later stages of healing, while the expression of genes associated with tissue regeneration increased after the initial inflammatory phase, approximately one week post-wounding, coinciding with scar tissue formation. The study provides new insights into the molecular and histological mechanisms underlying wound repair in red tilapia and suggests that Calendula gel may serve as an effective alternative to conventional antiseptics such as povidone-iodine for accelerating wound closure in fish aquaculture.

Keywords: Calendula, Histology, Gene expression, *Oreochromis sp*, Wound care

Introduction

Skin wounds and diseases are major constraints to the sustainable growth of many farmed aquaculture species, and maintaining healthy fish is a vital commitment to sustainable aquaculture (Jensen et al., 2015). Any skin disruption, such as mucus removal, scale loss, or deeper incisions, is negatively correlated with barrier function and disease resistance in fish (Sveen et al., 2018; Sveen et al., 2020). Superficial and deep wounds may develop into more prominent lesions, microbial invasion with complex pathology, and welfare issues for fish (Verma et al., 2017). The healing of deep wounds is more complex, and the process may last weeks to months, depending on the severity of the wound, fish species, and rearing environment (Richardson et al., 2013). Impaired wounds or delayed healing may result in increased instances of secondary infections, which often cause severe mortality. Wound re-epithelialisation is extremely rapid and recovery of skin wounds is of great significance for preventing the invasion of pathogens and maintaining the integrity of the surrounding tissue (Caraguel et al., 2016; Kumari et al., 2016; Xiong X-y et al., 2018; Sveen et al., 2020 & Greenspan et al., 2024). Wound healing is a complex and well-organized process in which physiological factors and immune mechanisms are involved (Przybylska-Diaz et al., 2013). Modelling of Zebra fish tissue regeneration (Caraguel et al., 2016) and dynamics of re-epithelialization in telost cichlid skin have been described (Sveen et al., 2020). Skin wound healing can be divided into a sequence of overlapping events: hemostasis, inflammation, proliferation, and remodeling. Water temperature, fish feed nutrition, and stocking density play key roles in the wound healing process through a highly regulated and suppressed inflammatory response (Vieira et al., 2011; Jensen et al., 2015; Sveen et al., 2018). The use of antibiotics in wound healing is not preferred because of the development of antibiotic resistance in bacteria and economic reasons (Önalán and Kankaya, 2024). Experimental applications on fish wound healing and the details of the stages of this healing process have been widely studied in farmed fish (Sveen et al., 2019 and Albaladejo-Riad et al., 2022). Topical antibiotic and anti-inflammatory drops and ointments for the treatment of corneal diseases can also be used in aquatic animals. Sterile saline or dilute chlorhexidine and povidine-iodine have been successfully used for the lavage of wounds in aquatic animals. Hydrogen peroxide is used in aquaculture as an immersion (bath) treatment against many disease-causing organisms, including external parasites, bacteria, and fungi, in other species and life stages of fish. The adverse effects of hydrogen peroxide also kill normal cells within the wound, including healthy skin and immune cells, and induce blood vessel formation. Bathing fish with an immunomodulatory β -glucan, a glucose-based polysaccharide product during wound healing, was found to have minimal effects on wound healing (Przybylska-Diaz et al., 2013; Schmidt et al., 2013; Schmidt et al., 2016). Bathing with β -glucan affects wound healing of silver catfish, *Rhamdia quelen* (dos Santos Voloski et al., 2019). A protocol to induce skin lesion by acetic acid injection (5%) was optimized and induced regional inflammation wound hyperpigmentation by recruiting pigment cells to the wound area (Xiong X-y et al., 2018). Efficacy of Juniperus root extract in healing experimentally induced incised wounds in chichlid, yellowtail acei *Pseudotropheus* sp. (Önalán and Kankaya, 2024). The probiotic *Shewanella putrefaciens* has been studied for skin healing of experimentally wounded gilthead seabream (*Sparus aurata* L.) (Chen et al., 2020). Zn-supplemented diets generally lead to increased chemokine expression during the first 24 h following wounding (Jensen et al., 2015). Oxytetracycline dihydrate (OTC) mediated feed on Nile tilapia (*Oreochromis niloticus*) and histopathological alterations in the kidney, muscle, and wound healing against pathogenic bacteria were investigated (Julinta et al., 2017). Silver nanoparticles enhance wound contraction in *Danio rerio* (Seo et al., 2017). Therapeutic asiaticoside on wound healing in Mrigal carp *Cirrhinus mrigala* (Verma et al., 2017). ZnSO₄ on the wound healing process in partially scaled common carp (El-Adl et al., 2024). Rifampicin, a broad-spectrum antibiotic used in the wound healing process, has a positive effect on wound healing rate (Yen et al., 2021).

Homeopathic medicines may have their own mechanisms of action and could offer an alternative approach to promoting healing in aquaculture without harmful chemical residues (Narayanan & Vidhya 2025).. The anti-inflammatory properties of *Calendula* have been demonstrated in animal models; however, its precise mechanism of action remains poorly understood (Silva et al., 2021). The major bioactive compounds identified in *Calendula* flower extracts include triterpenoids and flavonoids, particularly flavonol glycosides. Wound healing involves a complex sequence of cellular processes, including cell proliferation, differentiation, apoptosis, macrophage activity, myogenesis, and angiogenesis. These processes have been investigated in various fish species (Richardson et al., 2013; Schmidt et al., 2016; Vélez et al., 2017; Sveen et al., 2020; Okwuosa et al., 2021; Glover et al., 2013; Przybylska-Diaz et al., 2013; Bouyoucos et al., 2020; Pandey et al., 2021; Campos-Sanchez et al., 2021a; Ceballos-Francisco et al., 2017; Espinosa-Ruiz et al., 2021). The techniques that have been developed for eliciting cutaneous wounds in the zebrafish, including an economical method was reviewed (Greenspan et al., 2024). Inflammatory responses to injury mimicking infection using real-time gene expression analysed in carp (*Cyprinus carpio* L.) skin. Relative gene expression was analyzed in the skin samples of Gilthead Seabream (*Sparus aurata* L.) (Espinosa-Ruiz & Esteban, 2021). Gene expression patterns

indicative of active myogenesis were observed 16 days after injury in juvenile gilthead sea bream (*Sparus aurata*), suggesting ongoing muscle regeneration and angiogenesis accompanied by the formation of new muscle fibres (Otero-Tarrazón et al., 2023).

The present study was undertaken to evaluate the potential of the homeopathic medicine *Calendula 30* to enhance wound healing and muscle recovery in Red Tilapia (*Oreochromis sp.*). The study aimed to assess a cost-effective and safe therapeutic approach for promoting rapid wound closure with minimal adverse effects. Particular emphasis was placed on wound healing and the expression of genes associated with tissue repair and regeneration. *Calendula 30* was expected to support granulation tissue formation while minimizing concerns related to chemical residues. To our knowledge, this is among the first studies to investigate the effects of *Calendula 30* on wound healing and the expression of genes associated with tissue repair and regeneration in Red Tilapia.

Materials and methods

Ethical statement and approach

All experimental procedures were conducted in accordance with institutional guidelines for animal care and research. Prior approval for the use and care of live freshwater fish fingerlings was obtained from the institutional ethical committee (EC/NEW/INST/2023/TN/032), RVS Dental College and Hospital, Sulur, Coimbatore / comparable ethical standards under the project, SHORT TERM STUDENTSHIP IN HOMOEOPATHY (STSH) .

Maintenance of Experimental fishes

Selected fish species belong to the family Cichlidae, Red tilapia fish (*Oreochromis sp*) fingerlings (120 numbers) size 3-4 cm were procured from local farmers (procured from the fish hatchery, Barur Centre for Sustainable Aquaculture, Krishnagiri, Tamil Nadu) and transported to the experimental lab, RVS Homoeopathy Medical College and Hospital, Kumarankottam, Sulur, Coimbatore, INDIA. The fishes were accumulated in freshwater tanks (100 l) with adequate aeration and fed twice a day (commercial pellet feed of 38/8 crude protein and fat %) for four weeks before being procured for the experiments (Fig.1). On the day of the experiment, the fish were measured (length and weight), and the same size was secerated in different treatment tanks without feed.

Design of experiment

Four treatments with three replicates were used. Sample size: Eight fish per replicate. Experimental duration: 15 days

Sampling duration: Daily visual observation of wounded fish was carried out.

Further, microbial load, blood parameters, histology, and gene expression were analyzed in 0th, 5th 10th days interval (Fig.2).

GROUP I : Served as a normal control (C1)

GROUP II: Excision of wounded fish without treatment (T 1)

GROUP III : Excision for wounded fish treated daily with calendula 30 (T 2)

GROUP IV : Excision of wounded fish treated with povidone-iodine (T 3)

Inclusion criteria

Healthy fishes without any external injuries or infections were selected for the experiment.

Juveniles of similar size

Larger size fishes were selected.

Exclusion criteria

Unhealthy, infected, and stressed fish were excluded from the study.

Cut wound generation and antibiotic treatments

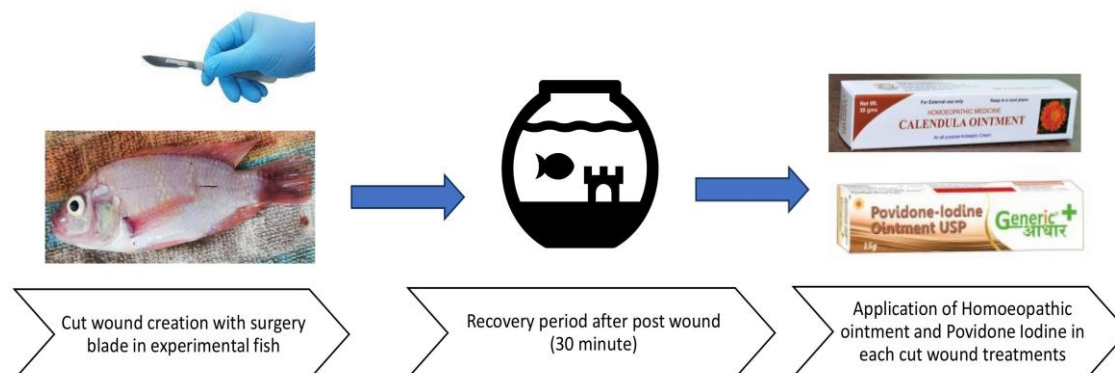
Surgery was performed on red tilapia fish with scales exposed on the surface of the epidermis. Fish were anesthetized with clove oil to create experimental wounds. The epithelial cut wound was created with a sterile surgical blade (No. 22) following standard procedures and ethical norms (Vergneau-Grosset & Weber III, 2021 and Greenspan et al., 2024). Lateral 10 mm incisions were made with a sterile scalpel on the tissue between the dorsal fin above the lateral line of the fish. The experimental procedures were applied subcutaneously, 15 minutes after the surgery. Before treatment, the blood was suddenly wiped-off (Fig.4).

Imaging and Quandification of Cut Wounds

Wound size were measured to determine the percentage difference in healed wounds before and after administering the homeopathic medicine.

Measurement of wound contraction

Visual observation of wound healing and the percentage of wound contraction were calculated. The wound size (mm) was recorded using a Vernir caliper and a graph sheet. Tissue layer formation was observed using a stereomicroscope. The study was initiated immediately after the experimental wound formation in the fishes. Each fish



(Medicine image credit: [https:// medilifeonline.shop](https://medilifeonline.shop))

was evaluated according to wound size and closure rate compared with the previous day. Differences between groups were correlated using wound closure time. The wound surface area was measured daily.

Sampling for Microbiology: Sterile swabs were taken to observe the microbial load from the wound site following the standard procedure (OIE, 2020). Random samples were swabbed from the cut wounded area using the serial dilution method and spread over the nutrient agar medium in Petri plates under aseptic conditions following the streaking method. The plates were incubated in B.O.D at $30 \pm 10^\circ\text{C}$ for 24h. Bacterial growth on nutrient agar plates was observed after 24h. Colonial morphology and microscopic appearance were observed by Gram staining.

Haematology analysis: Blood samples were collected to study haematological parameters following the standard protocol during wound healing (Linga Prabu et al., 2022). The fish were collected using a hand net with the least stress, transferred carefully into the vessel with an anesthetic solution (clove oil at a dose of 50 mg L^{-1}), and allowed to swim until they lost equilibrium to get sedated absolutely. Once the fish completely lost its equilibrium, it was removed and kept in soft wet turkey cloth to prevent mucous sloughing during sampling. It should be ensured that the fish did not show any movement; thereafter, the fish handled gently and therefore did not feel pain while collecting blood. Needles and syringes were selected according to the size of the fish. The needle should be sterile and changed for every fish to avert infection if the fish is required to rise further and clot less, thus preventing RBC rupture and hemolysis. For small-sized fish ($<30 \text{ g}$), a 26 G needle fixed in a 1 mL tuberculin® syringe would be ideal. The needle was rinsed with an anticoagulant (2.75% EDTA solution/heparin solution) to prevent blood coagulation during the course of blood collection. The needle was inserted beneath the scales humanely into the caudal peduncle area of the fish just below the lateral line at an angle of approximately 45° to the lateral surface in a cranial or ventral direction, until the needle touched the vertebral column. This can be felt by a hard impenetrable surface; thereafter, the syringe should be withdrawn slightly, approximately 1-2 mm, so that the blood vessel overlies the vertebral column and sampled. After blood collection, the fish were released into a recovery tank with fresh aerated aesthesia-free water and allowed to swim freely for 60 min.

Histology analysis: Wound tissue samples were collected and placed in neutral buffered formalin for 24 h for routine histology and 5-7 μm sections were stained with hematoxylin and eosin (Tissue samples given to private laboratory, Chennai, India for making histology slides). Wound healing was assessed according to the histological score, as described (dos Santos Voloski et al., 2019).

Sample preparation for Gene Expression analysis: Muscle tissue samples were collected and preserved in RNAlater and sent for further analysis (outsourced for further molecular analysis). Selected genes, such as tumor necrosis factor- α (TNF- α) and Transforming Growth Factor (TGF)- $\beta 1$, were assessed with quantitative PCR (qPCR) for the pattern of expression during the wound healing process in the control and treatment groups. Gene expression analysis was conducted using specific primer pairs (Schmidt and Jacob Günther., 2013; El-Adl et al. 2018).

Statistical analysis

The results of the experiment are expressed as mean $\pm$ standard deviation and were analyzed by one-way analysis of variance (ANOVA) using SPSS software to test the significance of differences among the experimental fish groups. Mean values were compared using Duncan's Multiple Range Test. A probability level of ≥ 0.05 was used to determine the significance in all cases.

Results

To assess the muscle wound healing properties of the homeopathic medicine Calendula 30 in red tilapia *Oreochromis sp*

Observation of Pre & post-wound process

1st day

Fish were anesthetized with clove oil to create an experimental wound, and lateral 10 mm incisions were made with a sterile scalpel on the superficial muscle tissue between the dorsal and caudal fins. Mucous cells were mainly present in the superficial layers. Red Tilapia skin had the typical organization of teleost skin and was composed of three well-defined layers: the epidermis, dermis, and hypodermis, which overlaid a fat layer that varied in thickness. The scales were enclosed within a scale pocket and composed of a mineralized external layer and a partially mineralized basal plate. The scale pocket was localized in the superficial dermis and projected into and was covered by a thin layer of the epidermis (Fig.3).

Re epithelisation

The first response to a deep wound is bleeding, and there is no evidence of blood clot formation in fish despite the active genes involved in hemostasis and blood coagulation after infliction. An amorphous substrate filling the wound was observed at T2. The initial reepithelialization process is believed to be independent of cell proliferation and inflammation. Epidermal cells began to cover the wound, and the first signs of inflammation were observed. A new epidermis with rounded epithelial cells is under development. The epidermis showed more vacuolization, less organization, and a less pronounced basal layer, suggesting delayed healing processes in T1. Skin regeneration involves re-epithelization followed by scale regeneration.

Inflammation follows Re-epithelisation

In the wound bed and inflammatory response, the recruitment of leukocytes and macrophages to clear the wound from damaged tissues drives the repair process. Initially, neutrophils remained behind the leading edge of the re-epithelizing epidermis, and later, more neutrophils and macrophages were recruited to the wound site. The number of neutrophils and macrophages peaked after wounding and gradually dropped over a 4 day period leaving some macrophages.

3rd – 5th days

Granulation tissue formation and wound contraction

The repaired tissue, also known as granulation tissue, consists of connective tissue, fibroblasts, myofibroblasts, immune cells, and small blood vessels. Tissue typically grows from wound borders, and with time, it replaces the damaged tissue. Granulation tissue drives wound contraction. The contracted area became elongated in the dorsoventral direction. On the 5th dpw, the new tissue was highly vacuolated and the superficial cell layer was weak (Fig.5). Organization of the basal layer was more developed along the wound boundaries, less organized in the wound center, and where the foundation was fluid as described (Jensen et al., 2015).

Alteration in skin pigmentation

In deep wounds, melanin granules are rapidly released, which slightly alters skin pigmentation. In T 2 and T3, there was hyperpigmentation of the wounded area when compared to T1, which indicated granulation tissue formation. Hyperpigmentation is caused by melanocytes infiltrating collagen-rich repair tissues.

10th day

The intensity of wound contraction was higher in T2 and T3 than in T1. By 10th day the entire surface was re-epithelized, and scale regeneration had occurred (Fig.6).

Observed on cellular response of wound healing process

Hemostasis

Immediately after the disruption of tissue integrity, platelets become adherent and clotting factors form a hemostatic plug to stop bleeding from the vessels. The blood vessels undergo vasoconstriction. Histamine is the primary mediator of the inflammatory vascular response.

Inflammation

In the early stages of inflammation, motile white cells actively migrate into the wound and start engulfing and removing cellular debris and injured tissue fragments. Initially, polymorphonuclear leukocytes dominated. As the transient phase of white cell migration ends, the granulocytes with a shorter life span and monocytes engulf and ingest cellular debris.

Proliferation and granular tissue formation

The hematoma within the wound is soon replaced by granulation tissue consisting of new capillaries and fibroblasts. The granulation tissue is mainly formed by proliferation and migration of the surrounding connecting tissue



Fig.1. Setup of Experimental tanks (Control and Treatments)



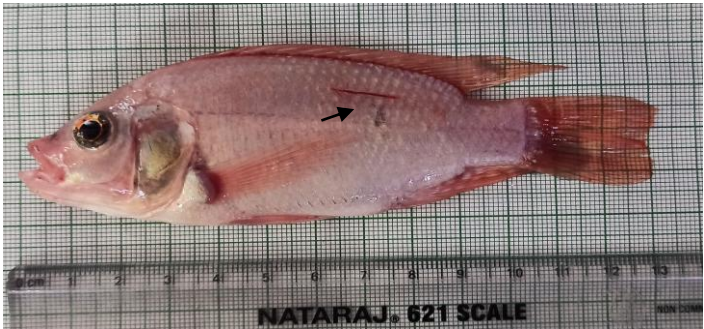
Fig.2. Experimental fish Red Tilapia fish *Oreochromis sp*

1st
Day of Post
Wound
(dpw)

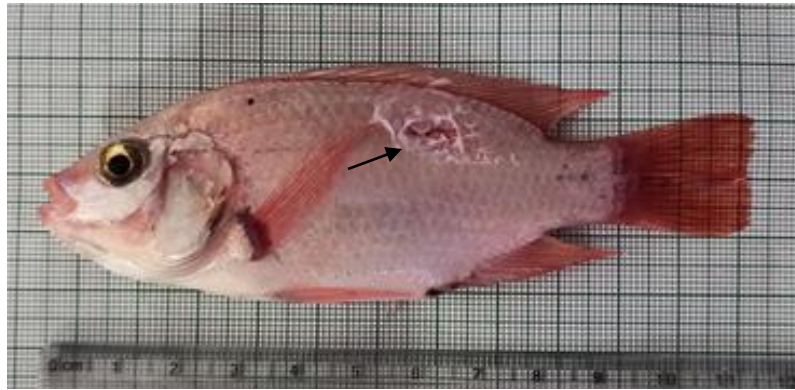
No cut



T-1
(No Medicine
Applied in the
cut wound)



T-2
(**Calendula 30**
ointment
applied in the cut
wound)



T3
(**Povidone**
iodine ointment
applied in the cut
wound)

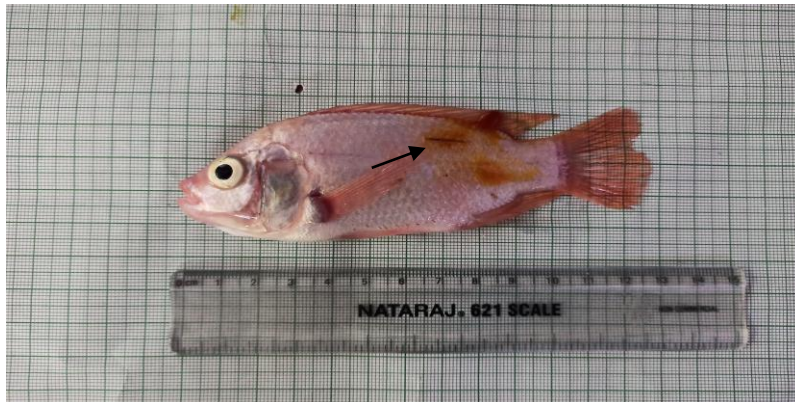


Fig.3. Creation of incision cut wound ; applied antibiotic (T3), Homoeo group (T2), No medicine (T1) arrows point to wound areas, scale bars (10 mm)

elements it is in fact composed of in the first instance by capillary loops and fibroblasts with a variable number of inflammatory cells. Initially, it is a highly vascular tissue that gradually turns into avascular scar tissue.

Granulated tissue consists of connective tissue, fibroblasts, myofibroblasts, immune cells, and small blood vessels. grows from the wound borders and replaces the damaged tissue over time.

Re epithelization

In skin wounds, the epidermis begins to thicken immediately adjacent to the wound edge. Marginal basal cells lose their firm attachment to the underlying dermis, enlarging, and begin to migrate into the wound. The fixed basal cells in a zone near the wound edge undergo rapid mitotic division (proliferation), the daughter cells migrate within 2 days, and the entire wound surface is re-epithelized. After bridging the wound defect, the migrating cells lost their flattened appearance and became more columnar in shape. Layering of the epithelium starts, and surface cells keratinize. Epithelization of the wound mainly occurs by the proliferation and migration of marginal basal cells lying close to the wound margin.

Wound Contraction

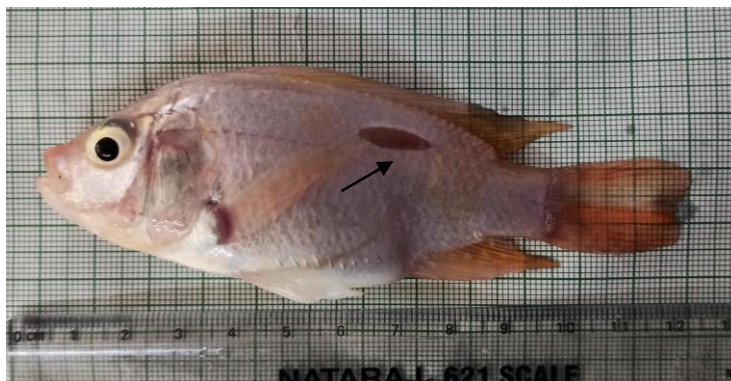
The wound contraction did not begin immediately, and approximately 3 to 4 days elapsed before the movement of the edges became measurable. This period, when no wound contraction was observed, is called the initial lag period. After this period, by day 10, the wound was reduced by approximately 80% of its original size. The wound position can also influence the contraction rate. The amount of contraction depends on the amount of skin available surrounding the wound to stretch over the wound.

Tissue Maturation

This is the final stage of the wound healing process. The transition from granulation to scar tissue occurs between 10 and 15 days post-injury. Finally, by 14 days post wounding, it is difficult to localize the former wound (Caraguel et al., 2016).

Effect of homeopathic medicine calendula in wound healing Calendula enhances collagen synthesis, promotes collagen production, and promotes wound healing. The calendula increases tensile strength and stimulates blood vessel

T-1



T-2



T-3



Representative photos of wound morphology

Fig 4. 5th Day of Post Wound (dpw) - Image analysis of wound areas

formation, supporting granulation tissue formation. The calendula reduces inflammation. Promoting a conducive environment for the healing calendula has antioxidant properties; it accelerates the wound healing process in T2 samples in which calendula ointment was applied (Fig.6). **Water quality analysis**

Water quality parameters, such as dissolved oxygen, temperature, pH, alkalinity, hardness, ammonia, nitrite, and nitrate, were assessed daily, and the results are reported as follows (Table.2)

Microbiology

Microbiological analysis was performed to determine the total plate count, total colony forming units, and bacterial strains present in the fish skin/wounds (Graph.2). The following bacterial strains, *Aeromonas* spp., *Enterococcus faecalis*, *Enterobacter* sp., *Bacillus cereus*, *B. thuringiensis*, *B. toyonensis*, *Pseudomonas aeruginosa* and *Lysinibacillus odysey* were present in the skin of the experimental fish presented in Table. 3.

Haematology analysis

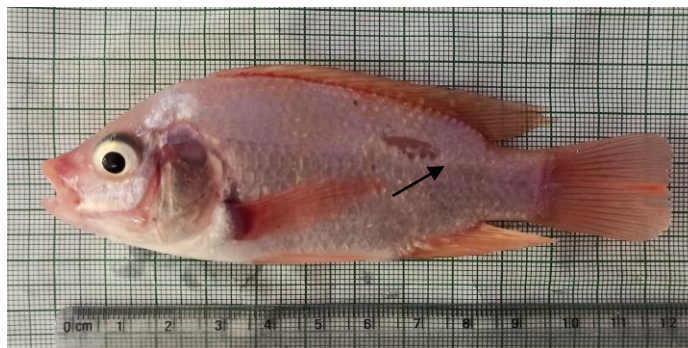
Hematological parameters such as red blood corpuscles (RBC), white blood corpuscles (WBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were analyzed in the blood samples collected from the respective treatment groups. Hemoglobin and white blood corpuscles showed significant differences ($P < 0.001$) among the

treatments, and no significant differences ($P>0.05$) were observed in the remaining hematological parameters, including RBC, HCT, MCV, MCHC, and MCHC values (Table.4).

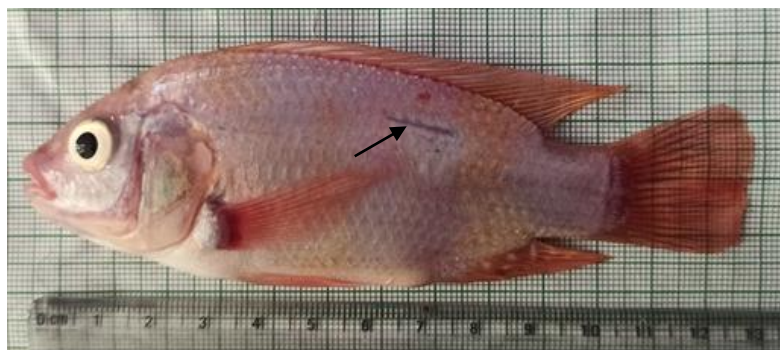
Histology analysis

Histology of muscle tissue was carried out in the wound healing area of red tilapia treated with Calendula medicine and the control group. In the control group the myofibrils were found normal and other treatment groups the myofibrils found slightly enlarged. The perimysium also widened in the treated groups due to cut/wounds compared to that in the control group.

T-1



T-2



T-3

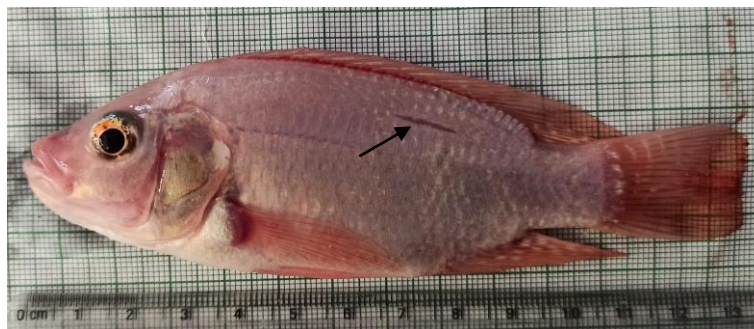


Fig. 5 Representative photos of wound morphology
10th Day of Post Wound (dpw) - Image analysis of wound area

Gene	Primer sequence (5'→3')	Referances
TGF-β1	Forward ACGTTTATTCCCAACCAAA Reverse GAAATCCTTGCTCTGCCTCA	Huttenhuis et al., 2006a Schmidt Jacob Günther., 2013

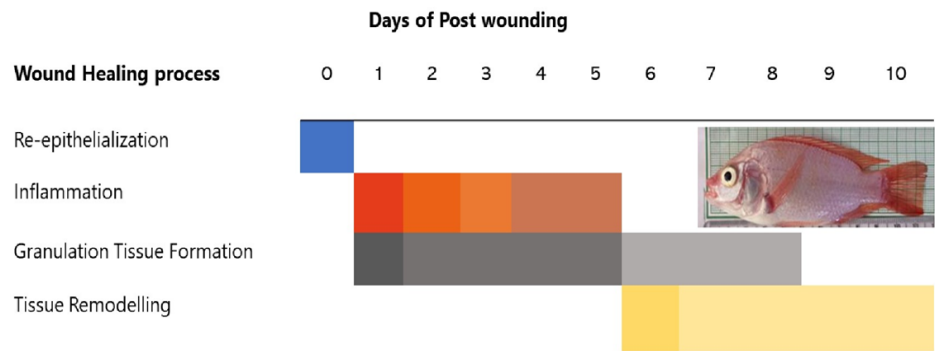
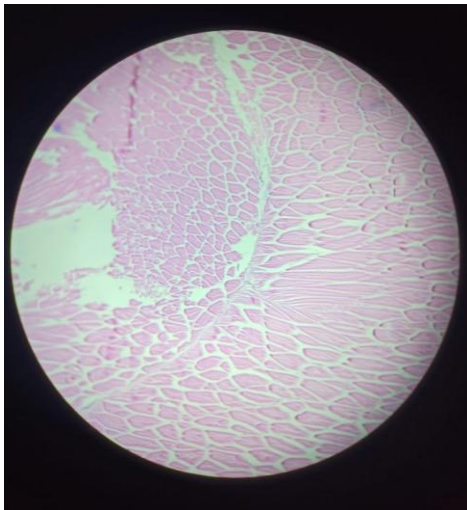
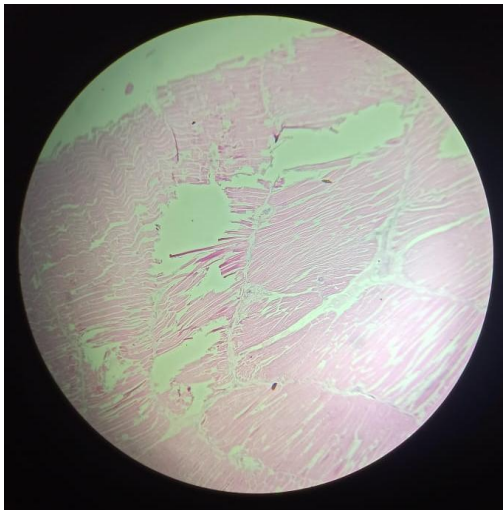


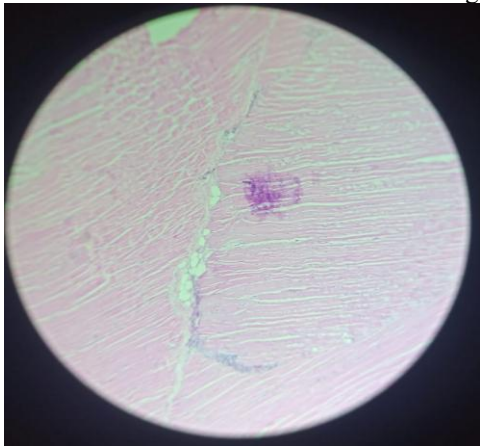
Fig 6 Flow chat for the days of wound healing process



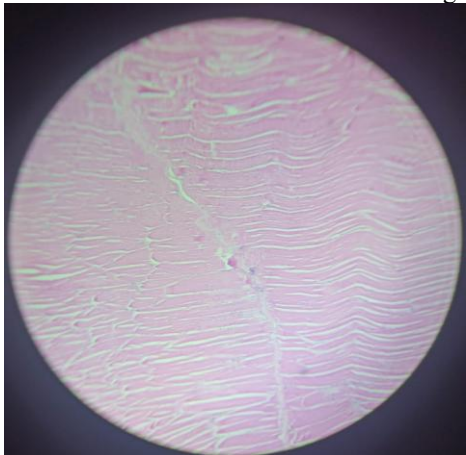
The muscle tissue section of control group



The muscle tissue section of T1 group



The muscle tissue section of T2 group



The muscle tissue section of T3 group

Fig 7. Histology slides of muscle tissue (10th dpw) in C, T1, T2 and T3

Table 1. Measurement of cut wound contraction

Treatments	Cut wound measurements (1th Day)		Cut wound measurements (5th Day)		Cut wound measurements (10th Day)	
	Length (mm)	Width (mm)	Length (mm)	Width (mm)	Length (mm)	Width (mm)
T-1	10±1.0	2.0±0.5	9.0±1.0	5.0±1.0	7.0±0.5	3.0±0.2
T-2	10±1.0	2.0±0.5	7.0±0.5	2.0±0.5	5.0±0.5	1.0±0.3
T-3	10±1.0	2.0±0.5	6.0±0.5	2.0±0.1	5.0±0.2	1.0±0.1

Random sampling size of treatments (n = 5)

Table 2. Water quality recorded during the experiments

Parameters	Control	T1	T2	T3
Temperature (°C)	25.1-29.4	25.6-29.5	25.4-29.6	25.4-29.4
pH	7.9-8.1	7.9-8.0	7.9-8.2	7.8-8.2
Dissolved oxygen (mg L ⁻¹)	6.2-6.8	6.5-6.7	6.4-6.9	6.3-6.8
Hardness (mg L ⁻¹)	230-265	225-255	220-245	230-245
Alkalinity (mg L ⁻¹)	250-270	260-275	270-290	260-285
Ammonia (mg L ⁻¹)	0.05-0.10	0.07-0.09	0.06-0.09	0.07-0.09
Nitrate (mg L ⁻¹)	0.30-0.40	0.40-0.50	0.35-0.45	0.40-0.45
Nitrite (mg L ⁻¹)	0.05-0.07	0.03-0.05	0.03-0.04	0.05-0.06

Table 3. The bacterial strains present in different treatment groups

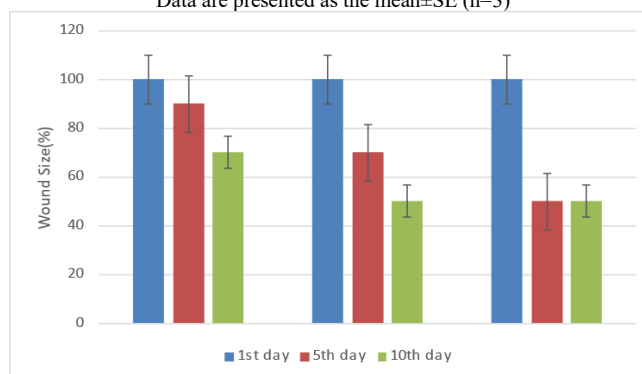
Bacterial strain	Control	T1	T2	T3
<i>Aeromonas</i> spp	+	+	+	+
<i>Enterococcus faecalis</i>	+	+	-	+
<i>Enterobacter</i> sp.	+	+	+	+
<i>Bacillus cereus</i>	+	+	+	-
<i>B. toyonensis</i>	+	+	+	-
<i>B. thuringiensis</i>		+	+	+
<i>Lysinibacillus odysey</i>	+	-	+	+
<i>Pseudomonas aeruginosa</i>	+	+	+	-

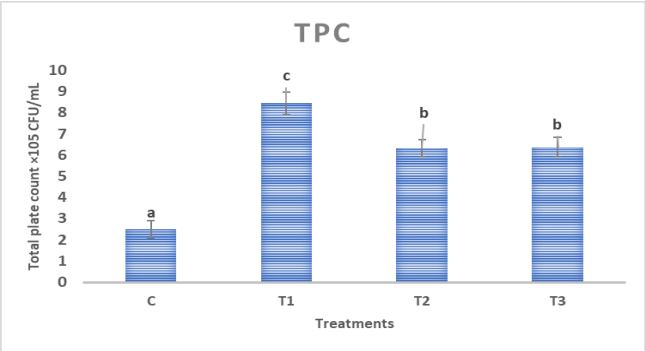
+ present; - absent

Table 4. Observed hematological parameters during wound healing process

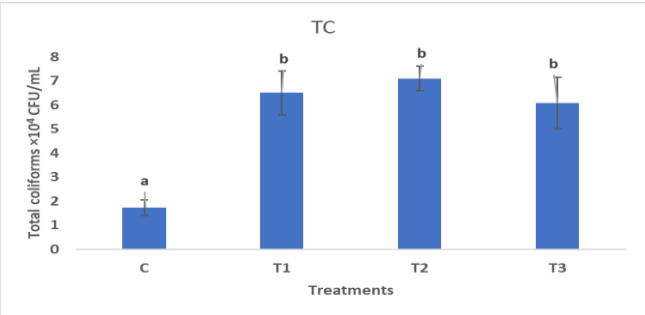
Parameter	Control	T1	T2	T3	P Value
Hb	9.53±0.12 ^b	8.28±0.23 ^a	8.50±0.11 ^a	8.50±0.06 ^a	0.001
WBC	5.32±0.08 ^a	6.23±0.31 ^b	7.46±0.17 ^c	7.26±0.12 ^c	0.001
RBC	3.93±0.14	3.63±0.06	3.66±0.15	3.61±0.21	0.465
HCT	27.76±0.47	26.90±0.51	27.23±0.24	27.26±0.29	0.523
MCV	70.70±1.51	74.10±2.40	74.60±3.21	76.14±5.12	0.710
MCH	24.30±0.99	22.80±0.65	23.30±1.25	23.74±1.62	0.832
MCHC	34.35±0.53	30.84±0.67	31.22±1.41	31.22±0.64	0.065

Data are presented as the mean±SE (n=3)

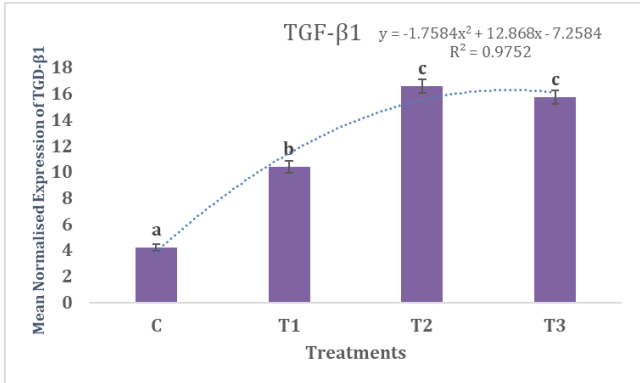
**Graph 1.** Percentage reduction in cut wound size in treatments vs. dpw (T1, T2, and T3)



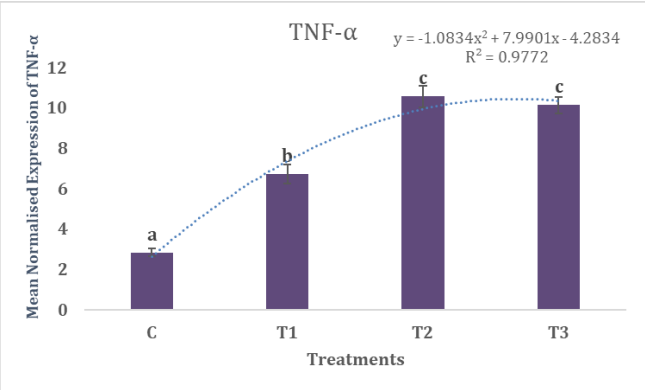
Graph 2 a. Total plate count observed in experimental fishes



Graph 2 b. Total plate count and Total coliforms observed in experimental fishes



Graph 3 a. Expression profile of TGF-β1 and TNF-α



Graph 3 b. Expression profile of TGF-β1 and TNF-α

Interstitial cells such as fibroblasts or capillary endothelial cells found in the perimysium were normal in all groups. Histology of day 10 dpw samples revealed a rapid repair process, with the epidermis already re-established (T2 and T3). Hence, within 5 days, the animals re-established their external barrier and protection to the environment, and 10 days after the epithelial wound, a thin regenerated scale was visible (Fig. 7). From a morphological perspective, the regeneration process in tilapia is similar to that described in the cichlid *Hemichromis bimaculatus*.

Gene Expression

To understand the relative gene expression in the wound healing process in red tilapia treated with Calendula. The expression of growth-related genes, such as transforming growth factor- β 1 (TGF- β 1) and tumor necrosis factor- α (TNF- α), was studied after 10 days of treatment. The expression of TGF- β 1 and TNF- α was upregulated in the T2 and T3 groups compared to that in the T1 and uncut control groups. However, maximum expression was observed in the T2 group (Graph.3).

Discussion

Wound Healing process

In the present study, the size of the measured cut wound were (10 ± 1.0 & 2.0 ± 0.5 mm) in all the treatments (Graph.1). Skin biopsies (5 mm) taken from behind the dorsal fin of Atlantic salmon post-smolts were followed over a 2 month period. The wound healing rate was quantified as the extent to which wound size decreased (Sveen et al., 2019). Rapid granulation tissue formation, with tissue already present at 2 dpw and maximum size at 4 dpw, resulted in Danio rerio (Richardson et al. 2013). Granulation tissue and scale development were prominent at 14 dpw in Atlantic salmon. The granulation tissue was gradually cleared over a six-day period. (Sveen et al. 2018; Sveen et al., 2019). Rai et al. (2012) reported that cyprinid Rohu (*Labeo rohita*) 5 mm wide and 2–3 mm deep cutaneous wounds were fully re-epithelialized after 4–6 hours (at 25°C). Proliferation, organization, and differentiation of the cells of the healing epidermis occur 9 to 48 h after re-epithelialization and are typically complete within 3 to 4 days. *H. bimaculatus*, cell proliferation peaked at 4 days post-injury and then decreased, with restoration of all cell layers completed by 6 days post-trauma.

Wound healing is a complex and well-organized process that can be roughly divided into three overlapping phases: inflammation, tissue formation, and remodeling, in which blood cells, soluble mediators, resident cells (fibroblasts, endothelial cells, and goblet cells), and extracellular matrix components are involved (Przybylska-Diaz et al., 2013). Fibroblasts are involved in the initiation of wound repair & regeneration promotion on cell proliferation and angiogenesis (Xiong X-y et al., 2018). Genes encoding the proinflammatory molecules interleukin-1 and cyclooxygenase-2 suggest that specific collagen motifs are involved in the regulation of inflammatory and healing responses in teleost fish (Castillo-Briceño et al., 2011).

In the present study, the superficial application of homeopathic Calendula gel improved the collagen regulation process at 1–5 dpw compare to the control group. The probiotic *Shewanella putrefaciens* showed that dietary administration of SpPdp11 at a dosage of 10^9 cfu g⁻¹ has a positive effect on skin wound healing in experimentally wounded gilthead seabream specimens. The finding stated that probiotics could help neovascularization and synthesis of matrix proteins at the wound site, and angiogenesis is a crucial process in normal wound healing, involving neovascularization at the wound site, which prevents the development of ischemic necrosis and stimulates the tissue repair process (Chen et al., 2020). The progress of re-epithelialization continued after 7 days of recovery and revascularization is critical for wound healing (Greenspan et al., 2024). Reactive oxygen species (ROS) are essential secondary messenger signaling molecules for many immune cells and appear to be important for coordinating the recruitment of immune cells to the wound site and effective tissue repair. The oxidative stress caused by continuous ROS production regulated the recruitment of immune cells and induced sustained pro-inflammatory cytokine secretion, which led to DNA damage and cell apoptosis, thereby affecting wound healing; image analysis demonstrated that the decrease in wound areas was detected macroscopically and confirmed in fish treated with homeoecomedicine. Re-epithelialization is executed and regulated by an equally complex signaling network involving the β family and insulin-like growth factors. Proteolytic degradation of the extracellular matrix (ECM) by (mmps) is also involved in re-epithelialization. The size of the wounded areas increased immediately after Ter wounding and then began to decrease as the day progressed, as observed in the present study. Costa and Power (2018) found that re-epithelialization of the epidermis continued for only 2 days in gilthead seabream skin; this finding was not observed in the present study, but a scare was noticed in the cut wound area after 10 dpw in the control group. Wound contraction does not occur in *Hemichromis* after scale and epidermis removal despite serious damage at the center of the wound. The wound surface was still visible after complete healing and was the same size as that immediately after wounding. *H. bimaculatus*, the tissues at the wound margin did not retract, but histolysis occurred first as a consequence of osmotic shock. Collagen

matrix deposition in *H. bimaculatus* is particularly important from the fourth day after surgery onwards, as observed in the present study in the treatment and control groups. Fibroblast-dependent angiogenesis is favorable for wound healing. Macrophages are known to promote fibroblast migration towards wounded regions and enhance proliferation by producing growth factors and cytokines. Wound healing involves migration of epithelial cells (keratocytes) from the surrounding tissue to cover the wound surface (Sveen et al., 2019). The type of scale used was correlated with the wound healing rate, as observed in the present study. Wound healing was faster in two fish species of the family Cyprinidae, which have scales exposed on the surface of the epidermis, than in three fish species of the family Cobitidae, which have scales embedded in the dermis. The differences in the wound healing rate could be attributed to host factors, including genetic factors, the migration and functions of keratocytes and immune cells, and cell proliferation (Richardson et al., 2013; Sveen et al., 2022).

In the present study, the healing process was dominated by hemostasis, acute inflammation, and epidermal repair on the 5 dpw. The studies by Greenspan et al., (2024) shows that four days after injury, granulation tissue formation was observed, followed by increased collagen deposition between 4 and 7 days after injury. Scale regeneration was evident by 10 days, accompanied by the gradual resolution of granulation tissue. By 15 days, the tissue layers had begun to reform, and normal tissue thickness was restored by 20 days after injury, however 14 days post wounding (dpw) in Atlantic salmon post-smolts (Sveen et al., 2019). Owing to the severity of the wounds inflicted by the biopsy punch and resultant variances between individual wounds, it was difficult to assess measurements by quantitative histology during the first 72 h, necessitating subjective morphological assessments during this phase. After 3 days, all the examined wounds were closed, but the epidermis was more structured. Subjective and quantitative histology confirmed that temperature has a large impact on the complex process of wound healing (Jensen et al., 2015).

Application of medicine

The medi-care groups T2 and T3 exhibited a quicker healing process than the control. Juniperus root extract has been shown to produce positive results (Önalan and Kankaya, 2024). Differences in the composition of skin between fish species may underlie the varied wound-healing rates. The presence of club cells varies among fish species, and club cells are presumed to perform protective functions against external stressors, including mechanical wounds. Other factors, including the difference in the density of club cells, the response of club cells upon mechanical wounding, and the contents of the club cells, may play a role in varying wound healing rates (Pandey et al., 2021). Rifampicin treatment changes the composition of microbiomes in both Korean bullheads and Chinese bleak skin (Yun et al., 2021). In the initial stage, keratocytes derived from the intermediate layer of the epidermis move quickly to cover the wound area, followed by inflammation. Neutrophils and macrophages are recruited to the wound area to induce inflammation and activate growth factor signaling, which promotes cell proliferation and granulation tissue formation. Granulation tissue forms along the wound borders and replaces the damaged tissue (Yun et al., 2021). Juniperus root extract in healing experimentally induced incised wounds in yellowtail acei *Pseudotropheus* sp. Scarring of the wound was observed on the 6th day, and in the root extract group, wound healing was completed on the second day. Wound closure time, morphology, malondialdehyde content, glutathione S-transferase activity, changes in gene expression levels, non-dispersibility of the extract in an aqueous environment, and retention on the wound were analyzed (Önalan and Kankaya, 2024). The repair of dermal and muscle structures took much longer than that of the epidermis. Rapid differentiation of the epidermal basal layer cells was observed during the re-epithelialization process after wounding of the cichlid fish. The results of the present study confirmed that the degree of wound healing was promoted by the application of homoeomedicine compared to the control and was similar in OTC-medicated feed, which was more prominent during the treatment period (Julinta et al., 2017). These chemotherapeutics provide effective, short-term anti-infective treatment during wound care but are eventually diluted into the aqueous environment upon release. Oral therapy can be accomplished using commercially available medicated pellets, medicated gel diets, injection or loading of prey food items, and gavage medication. Bath treatment is typically a shorter period of exposure to a more concentrated chemotherapeutic agent in water than prolonged immersion water therapy. The homoeomedicine Calendula 30 gel application to cut wounds in red tilapia fish is effective for a short period without any adverse effects on the fish and environment.

Microbiology

This study found that bacterial strains such as *Aeromonas* spp., *Enterococcus faecalis*, *Enterobacter* sp., *Bacillus cereus*, *B. thuringiensis*, *B. toyonensis*, *Pseudomonas aeruginosa* and *Lysinibacillus odysey* were present in the skin of the experimental fish. Wound healing rates vary between fish species, and the composition of microbiota is correlated with the wound healing rate (Yun et al., 2021). This suggests that host factors impede the wound healing process and that the presence of specific microbiota can facilitate or inhibit the wound healing rate. In the present study, microbiology results showed that The TPC was lower in the control group (2.5×10^5 CFU/mL) and higher in the T1 group (8.43×10^5 CFU/mL). TC was found to be lower in the control group (1.73×10^4 CFU/mL) and higher in the T2

group (7.1×10^4 CFU/mL). Verma et al. (2017) found that skin mucus plays an important role in protecting fish against microbial invasion. The epidermal cells spread on the wound surface, which is composed of two different matrices: the remains of basement membrane materials covering the scale-pockets and collagen fibrils of cut dermal strips. Although both matrices favor cell spreading and attachment, migrating cells show different behaviors. The basal layer cells, which spread on the substrate, and the superficial layer cells, which protect the epidermis, first differentiate.

Haematology

In the present study, hemoglobin and white blood corpuscles showed significant differences ($P < 0.001$) among the treatments, and no significant differences ($P > 0.05$) were observed in the remaining hematological parameters (RBC, HCT, MCV, MCHC, and MCHC). Fish sampled 7 days post-wounding had significantly decreased serum aspartate aminotransferase levels and increased albumin/globulin ratios. Immune cells secrete serum globulin (Chen *et al.*, 2020).

Histology

Histological analysis was performed to confirm that wound healing in each group was completed at 10 dpw. Similar to the control, which showed clear separation of boundaries between the epidermis, dermis, and red muscle, the wounded side also had distinguishable layers; however, the dermis was thickened, and the skin structure was considerably disorganized. Tissue damage was assessed using a score ranging from 0 to 6, depending on the degree and extent of damage based on the scale (Julinta et al., 2017). However, this was not performed in the present study because of the undefined readings of the slides. Sveen et al., (2019) observed that more adherent mucus was correlated with altered transcription of glycosyltransferases, indicating different properties and functions of mucus during the wound healing process. Histological studies have revealed that skin and scales regenerate rapidly in fish when they are lost or damaged. Histological analysis of the skin/scales revealed that re-epithelization and formation of the scale pocket occurred (Vieira et al., 2011).

Gene Expression

In the present study, the expression of growth-related genes such as transforming growth factor- $\beta 1$ (TGF- $\beta 1$) and tumor necrosis factor- α (TNF- α) was studied after 10 days of treatment. However, maximum expression was observed in the T2 group. The expression levels of stress proteins in the skin of the wounded flank of the wounded fish mainly increased at 7 dpw (Espinosa-Ruiz and Esteban, 2021). To determine the effectiveness of Juniperus root extract on wound healing in *Oncorhynchus mykiss* fish, tissue samples ($n=2$) were taken at the end of the first day and on the seventh day of the trial in the trial groups to determine the change in gene expression before and after wound healing. Gene expression analyses performed on samples taken from muscle tissues in the wound area showed that genes known to have a strong effect on the skin were expressed faster in a short time during the wound healing process in individuals treated with the extract (Önalán and Kankaya, 2024). During the healing process of the wound created in post-smolt *Salmo salar* fish, it was determined that the expression of immune genes was high at the beginning, decreased after two weeks, and inflammation in the wound area decreased and approached the level of intact skin (Sveen et al. 2019). SpDp11 inclusion in fish feed facilitated wound healing and re-epithelialization of the damaged skin and alleviated the inflammatory response in the wound area by intensifying the antioxidant system, enhancing neovascularization, and synthesizing matrix proteins in the skin wound sites of fish. The gene expression of igf-1 and tgf- $\beta 1$ in the SP group was significantly enhanced compared to the values of the compared to the control group. (Chen *et al.*, 2020). Thus, TGF- β is present in the early stages of wound healing and is one of the most studied molecular factors in wound healing (Schmidt *et al.*, 2013). Isoforms have been described in fish, although only TGF- $\beta 1$ and -2 from carp. TGF- $\beta 1$ is one of the most potent pro-fibrotic cytokines. It attracts neutrophils, macrophages, and fibroblasts to the wounds. Induction of fibroblast ECM production. TGF- $\beta 1$ has also been found to induce leukocyte apoptosis, which is important during the transition between different wound-healing phases. TGF- $\beta 1$ is incriminated in several phases of the wound healing process, including proliferative, regulatory, and inductive processes. The present finding proves that the expression level of TGF- $\beta 1$ showed a stepwise increase by increasing the concentration of Zn in an immersion water bath (El-Adl, et al., 2024). In the present study, the expression of TGF- $\beta 1$ and TNF- α was upregulated in the T2 and T3 groups compared to that in the T1 and uncut control groups.

Conclusion

The results presented in this study suggest that superficial application of Calendula and Povidine-iodine gel fastens the cut wound healing process in Red Tilapia, which is a short-term anti-infective treatment during wound care. Overall, Calendula gel represents a promising, safe, and cost-effective alternative for wound management in aquaculture. Future research should investigate the underlying cellular and molecular mechanisms using additional immune-related genes, antioxidant biomarkers, transcriptomic analyses, and other omics-based approaches to further validate its therapeutic potential.

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Ethical Compliance: All procedures performed in the studies involving in accordance with the ethical standards of the institutional ethical committee (EC/NEW/INST/2023/TN/032), RVS Dental College and Hospital, Sullur, Coimbatore / comparable ethical standards.

Ethical clearance

The present research does not aim to standardize homeopathic medicine for human use or any other livestock intended for fish health management. It is used by fish to promote granulation tissue formation by homeopathic means.

Data Access Statement: Research data supporting this publication are available and shared on the reference basis.

Conflict of Interest declaration

The corresponding author and co-author declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this research paper and subject matter or materials discussed in this manuscript.

Author Contributions: Prassana Devi Narayanan :PDN contributed to the experimental design, implementation of the research, writing of the manuscript, conceived the original, supervised the project and Iffath F :IF conducted experiments and analysed the results.

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