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CUTANEOUS WOUND HEALING BY *VERNONIA ARBOREA* EXTRACTS IN ADULT ZEBRAFISH MODEL

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Keywords:

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ABSTRACT: Cutaneous wound healing starts with an acute inflammatory phase marked by secretion of pro-inflammatory mediators that enhance infiltration of leukocytes with a peak in the first 24 to 48 h. This resolves to enable the continuation of other phases in series, namely, the proliferative, re-epithelialization, vascularisation, and tissue remodelling phases. The study uses a mechanical device, for the first time, to create a mechanical cutaneous wound in adult Zebrafish to simulate mammalian cutaneous wound. Topical application of 0.5% ointment of a fraction from hexane leaf extract of *Vernonia arborea*, accelerated wound healing in the developed model. The phytocompound in the fraction modulates the inflammation kinetics, increasing initial inflammation at 24 hrs by expediting neutrophil infiltration, three folds more than the untreated model. The resolution of inflammation was rapid in the experimental group after 3 dpw compared to the untreated control resulting in speedy proliferation and migration of keratinocytes and three times faster wound closure, measuring up to 92.3% as compared to 95.3% in the positive control group. The fraction also exhibited an anti-oxidant role and prevented the oxidative damage of wound tissue, with 30-40% higher granulation tissue weight than the povidone-iodine standard treatment. The extracellular matrix formation was found to be enhanced, marked by a two-fold increase in the expression of tissue markers like hexuronic acid, hydroxyproline, and hexosamine. The results were analogous to the wound healing process in mammals, making the phytocompound a potent topical wound-healing agent that may be tested in preclinical and clinical trials.

INTRODUCTION: Among the wounds that result in lack of structural and functional communication between the cells forming the tissue, those that result in significant blood loss and those that do not heal with time need a clinical intervention [1]. The delay in healing is influenced by a lot of factors like depth of the wound, diabetic condition, microbial colonization of the wound, immune-compromised conditions, malnutrition, and others [2].

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Screening for wound healing potency of natural compounds with antimicrobial properties warrants a convenient model system. Zebrafish have been successfully used in wound healing research over the last decade, establishing around 14 different model systems [3]. Most of them are regenerative models showing fin amputation or laser wounding and larval studies.

The present study aims to establish a mechanical cutaneous excision wound in adult Zebrafish to simulate mammalian excision wound models [4]. The model would be a good alternative to replace earlier reported higher animal models to screen for bioactivities. The mechanical cutaneous wound provides possibilities to study various wound healing parameters, reducing the number of

animals needed for such research. Compared to the laser wounding procedure, the mechanical wounding of fish requires a simple reproducible tool. This tool was tested for screening of wound healing properties of *Vernonia arborea* (family: Asteraceae) leaf extracts used traditionally [5, 6]. Therefore, a few potent hexane fractions showing excellent activity against selected wound pathogens were assayed in this model [7]. The wound healing property of the bioactive fractions was estimated in terms of physical wound closure, neutrophil infiltration to mark onset and resolution of inflammation, migration of cells, formation of granulation tissue, analysis of oxidative markers, and tissue markers [8, 9]. The cutaneous wound in the established model simulates mammalian healing patterns through the regular series of overlapping phases, inflammatory, proliferative, and tissue remodelling.

MATERIALS AND METHODS:

Zebrafish Maintenance: Healthy adult wild type Zebrafish stock obtained from farms, Kolathur, Chennai were maintained routinely [10]. The experimental group had a 50:50 mixture of male and female fish.

Establishing Zebrafish Wound Model: A quick circular cutaneous excision wound was created with a semi-automated mechanical device with calibrated dimensions designed for the purpose. This mechanical device was driven at a constant speed with a DC wiper motor. The spring-operated nail (blunt-ended – 3 mm) was customized in position according to the variations in the size of the fish. The selected fish were weighed and anaesthetized with Tricaine (MS-222-Sigma). The fish were positioned manually on the wounding device, and the height of the fish from the base was adjusted using metal plates of defined thickness to reach a position with a standard distance from the nail. Wounds were made along the dorsal surface of the fish.

Preparation and Treatment of the Wound Model with Bioactive Formulation: Four fractions from the hexane extract of *Vernonia arborea* leaves, F10, F26, F28 and F30 were selected for assessing wound healing properties based on their antimicrobial profile [7]. An ointment for topical application on the wound area was prepared using white petroleum base warmed to 50 °C and mixed with extracts to yield 0.5% w/w formulation and refrigerated until use [11]. The fish received topical application of the extract ointment preparation (one layer to cover the wound area) and immediately transferred to the recovery tank. Groups I to IV with 20 fish each received the four selected fractions. Group V, positive control, received standard wound-healing drug formulation (Povidone-iodine ointment 0.5% w/w); Group VI, negative control, received no treatment; Group VII, vehicle control, received the vehicle base (white petroleum). The wound models were observed during different stages of the treatment time, 0, 1, 3, 5, 7, and 10 days post wounding (dpw). Replicates were maintained for each group.

Determination of Percentage Wound Contraction: The size of the wound of the treated fish was measured on 5, 7, and 10 days post wounding (dpw), and the percentage of wound closure on day 10 was calculated as follows [12]

Percent wound contraction = (healed area/ total wound area) x 100

Histochemical Staining of Tissue Sections: At their respective observation time, the treated fish were euthanised using Tricaine and fixed in 10% neutral buffered formalin. The fixed fish underwent paraffin embedding and microtome sectioning.

The sections were stained with haematoxylin and eosin stain and analyzed further [13]. The tissue histomorphology was compared for the 7 groups of treated fish for the following parameters

Neutrophil Infiltration Assay: The H&E stained tissue sections were observed for the neutrophil population at the wound site during the inflammatory phase. The neutrophil population at 1 and 3 dpw were recorded and analyzed [14].

Reepithelialisation and Granulation Tissue Formation: The degree of migration of the cells to the wound site were observed in the H&E stained tissue sections and recorded at various time intervals of treatment as mentioned in the study design 0, 5, 7 and 10 dpw. Migration of keratinocytes and epithelial cells near the wound margin was recorded. This observation gave the rate of reepithelialization. Granulation tissue formation was observed in terms of the appearance

of the restratified epithelial layer along the wound surface [15].

Biochemical Markers of Healing:

Tissue Homogenate Preparation: The wound tissue was excised and homogenized with an appropriate volume of 0.25 M sucrose using a mortar and pestle. The homogenate was centrifuged at 700xg for 10 minutes [16]. The supernatant obtained was again centrifuged at 10,000 rpm for 20 min and stored for biochemical analyses.

Total Protein Content of Tissue Sample: The total protein content of the tissue sample was estimated by Bradford method using bovine serum albumin of known concentrations as standard [17].

Granulation Tissue Weight: The granulation tissue collected from the models on day10 pw was weighed and recorded as milligram per gram body weight of the fish [12].

Oxidative Markers of Healing:

Reduced Glutathione Estimation: Tissue homogenates (50 µl) from 5 and 7 dpw fish from each treatment groups were estimated using Ellman's reagent and calculated in comparison with the standard. The amount of GSH was expressed in µmole/mg tissue protein [18].

Malondialdehyde Estimation: Fifty microlitres of the tissue supernatant was prepared from each treatment group 10 dpw and estimated for malondialdehyde concentration with trichloroacetic acid and thiobarbituric acid. The thiobarbituric reactive species was read at 535 nm, and the result is expressed in nMole/mg protein [19].

Tissue Markers of Healing:

Hydroxyproline Estimation: The tissue was excised, weighed, and dried in an oven at 60°C to 70°C for 12 to 18 h and the dry weight was noted. The hydroxyproline concentration in the sample was estimated using a standard curve according to Woessner [20].

Hexosamine Estimation: The tissue dry weight was noted as described earlier. The concentration of hexosamine in the sample was estimated using Ehrlich's reagent (*p*-dimethylaminobenzaldehyde). The red color developed was read after 30 minutes at 530 nm [21].

Hexuronic Acid Estimation: The tissue homogenate was treated with diphenyl reagent following established protocol. The reaction was recorded by reading absorbance at 530nm. Hexuronic acid standard was used to estimate the concentrations in sample [22].

Statistical Analysis: The collected data were analyzed with IBM.SPSS statistics software 23.0 version. To describe the data, mean & SD were used. To find the significant difference in the multivariate analysis, the Kruskal Walli's test was used, followed by the bivariate analysis and the Mann-Whitney U test. In both the above statistical tools, the probability value of 0.05 was considered significant.

RESULT AND DISCUSSION:

Mechanical Cutaneous Wounding in Zebrafish Simulates Mammalian Wound Inflammatory Kinetics: Circular cutaneous wound with an outer diameter of about 3 mm and a central depth of about 2 mm was created with the mechanical device **Fig. 1**.

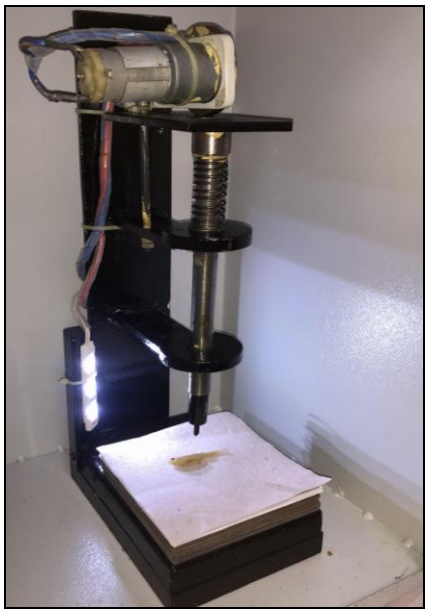


FIG. 1: SEMI-AUTOMATED DEVICE WITH DC WIPER MOTOR DESIGNED TO CREATE CUTANEOUS WOUND IN ADULT ZEBRAFISH

The wound destroyed continuation in the epidermis, scales, and extended upto the dermal layer as indicated by the histochemically stained tissue sections **Fig. 3**. In reports of laser-induced full-thickness cutaneous wounds, a similar loss of epidermal and dermal layers, including the scales,

was observed and the development of neoepidermis in such wounds was observed early, indicating wound closure independent of inflammation [4]. On the contrary, **Fig. 4** presents the natural increase and doubling of neutrophil populations at the wound site from 0 to 3 dpw indicating inflammation (untreated control and vehicle treated control) in mechanical wound model kinetics.

The re-epithelialisation and granulation tissue formation were observed 5-7 dpw and maximum wound closure being observed around 10 dpw. This is proposed to be comparable to the natural wound healing process in mammals.

Percentage Wound Contraction Over Time: The decrease in epithelialization time determines the rate of wound closure. The rate of wound closure in the model treated with just 0.5% of fraction 10 showed 3-fold better contraction than the untreated model. On day 10 pw, the maximum closure rate was found to be 92.3% in the F10 treated group compared to 95.33% in the standard group and 30% in the untreated group **Table 1, Fig. 2**.

TABLE 1: WOUND CLOSURE OBSERVED WITH TEST FRACTIONS F10-F30 [DAYS POST WOUNDING- DPW] IN ADULT ZEBRAFISH MECHANICAL WOUND MODEL [N=20]. FRACTIONS 10 AND 28 SHOWED WOUND CLOSURE SIMILAR TO THAT OF POSITIVE CONTROL

Wound diameter [mm] [mean ±SD].							
Observation time	**Test fractions [0.5% w/w]**				**Positive control**	**Untreated control**	**Vehicle control**
	F10	**F26**	**F28**	**F30**	**[PC]**	**[UC]**	**[VC]**
0 hrs	2.95±0.10	2.93±0.17	3.05±0.08	3.06±0.08	3.06±0.08	3.06±0.08	3.06±0.08
5 dpw	2.03±0.10	2.06±0.12	2.36±0.05	2.06±0.05	2.03±0.05	3.06±0.08	2.6±0.06
7 dpw	1.3±0.10	1.3±0.08	1.75±0.05	1.6±0.06	1.1±0.07	2.56±0.05	2.4±0.06
10 dpw	0.23±0.05	0.39±0.06	0.29±0.06	0.4±0.04	0.14±0.04	2.1±0.06	1.76±0.05
% Wound closure							
WC % 10 dpw	92.3	88.3	90.33	87	95.33	30	41.3

Treatment of Swiss albino mice mechanical wound model with 5% ointment of methanol extracts of *Acanthus polystachyus* showed 80% wound contraction [23]. Myricetin, isolated from *Tecomaria capensis* showed enhanced wound closure up to 98.76%, when applied topically to wounded rats at 20% concentration, compared to untreated rats with 67.35% closure [24]. Asiaticoside, from *Centella asiatica*, at a concentration of 1 mg/ml was found to significantly increase cellular proliferation and reduce apoptosis in epidermis and dermis in *Cirrhinus mrigala* excised wound created using biopsy punch[25]. Fifty percent of the ethanolic leaf extract in 200 mg of hydrogel of *Vernonia scorpioides* was found to improve regeneration and organization of the healing tissue in the mechanical wound excision model of guinea pigs though wound contraction time was not better [26].

Several natural products have been tested for wound healing potency in Zebrafish wound models over the years. Panax ginseng extracts showed angiogenic properties at 500µg/ml concentration in Zebrafish embryos [27]. Ginsenoside from the same plant has been studied for tissue regeneration in fin amputation model of Zebrafish larvae. The compound possessed anti-inflammatory activity with no change in tissue regeneration at 120 µM concentration [28]. Ethanolic extract of propolis was shown to increase caudal fin regeneration in hyperglycemic fin amputation model of Zebrafish at 15 ppm [29]. Silver nanoparticles of *Spirulina maxima*-derived pectin nanoparticles have been tested on the laser-induced wound models of Zebrafish. Both were found effective at 50 µg/ml concentration [30, 31].

The effect of neem leaves on wound closure of an injured Zebrafish was studied by Athiroh *et al*. [32]. Treatment with neem leaf slice and drops at 0.5, 1, and 2 g concentration was shown to cause wound shrinkage better than the untreated control.

The effectiveness of F10 and F28 fractions might be due to the enhanced early clearance of microbes and wound debri during the inflammatory phase and reduction in neutrophil population during later stages, preventing tissue damage that could extend epithelialization time **Table 1; Fig. 3**. As reported earlier, F10 fractions showed good antibiosis against five wound pathogens at 31.5 to 125 PPM concentrations [7].

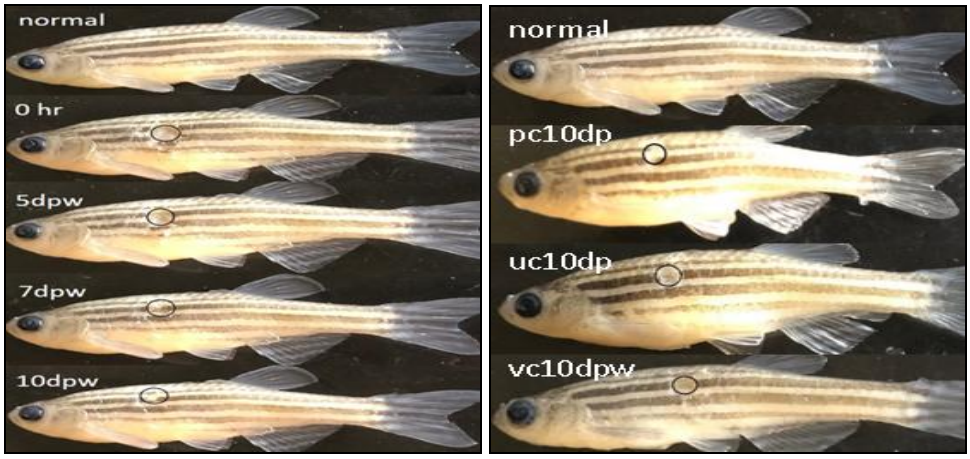


FIG. 2: ADULT ZEBRAFISH MECHANICAL WOUND MODEL [N=20] AFTER TREATMENT WITH F10 (0.5%), SHOWING WOUND CLOSURE ON 5, 7 AND 10 DPW. PC-POSITIVE CONTROL, UC-UNTREATED CONTROL, AND VC-VEHICLE CONTROL WERE SHOWN AT 10 DPW. MAXIMUM WOUND CLOSURE WAS OBSERVED IN F10 TREATED MODELS IN COMPARISON WITH THE POSITIVE STANDARD. SCALE BAR, 3 MM

Neutrophil Infiltration at the Wound Site: The elevated infiltration of the neutrophils marks the onset of the inflammatory phase. The recruited neutrophils clear the early microbial load and debris at the wound site [33]. In this study, the F10 (0.5%) treated fish showed a 3-fold increase in neutrophil population at 24 hrs and a drastic decrease after 3 dpw compared to the untreated control group **Fig. 3, 4**. Treatment with 20% crude extract of *Vernonia scorpioides* on excisional wounds in mice exhibited similar profile of inflammatory cells with an increase and then decrease during the observation periods of 3, 7 and 14 days [34]. In addition to the pathogen-evading role, neutrophils also directly affect angiogenesis, cell proliferation, and normal collagen deposition [35]. Upon recruitment, the neutrophils degranulate cytotoxic granules and reactive oxygen species to destroy the microbial load at the wound site. This damages the neighbouring host tissue if not resolved, resulting in impaired healing. Naturally, these neutrophils are phagocytosed by the macrophages resolving the inflammation [36]. The apoptotic neutrophils, stimulate the tissue macrophages to become efferocytotic to establish tissue homeostasis. In addition, neutrophil retrograde migration from the injury site to the vasculature was found to be contributing to the resolution of inflammation, as studied in the transgenic Zebrafish model [37]. It is proven that the treatment with fraction 10 increases neutrophil recruitment in the early stages and promotes clearance of the microbial population to prevent tissue damage and invasion[7].

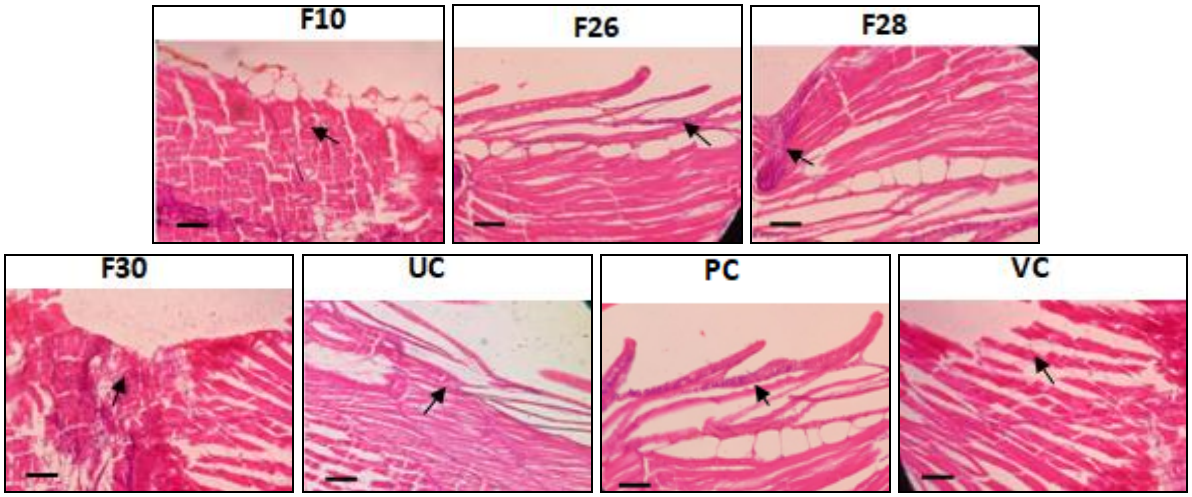


FIG. 3: H&E STAINED TISSUE SECTIONS AT 1DPW IN ADULT ZEBRAFISH MECHANICAL WOUND MODEL, SHOWING NEUTROPHIL INFILTRATION AT THE WOUND SITE, INDICATING ONSET OF WOUND INFLAMMATION. TREATMENT WITH F10 SHOWED 1.3 TIMES MORE NEUTROPHIL POPULATION THAN THE POSITIVE CONTROL. SCALE BAR, 30 µM

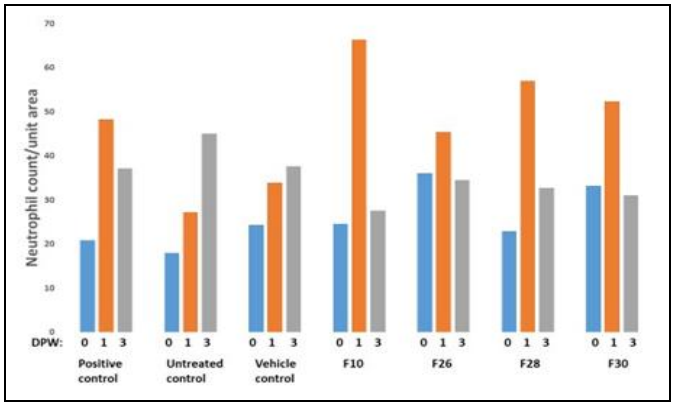
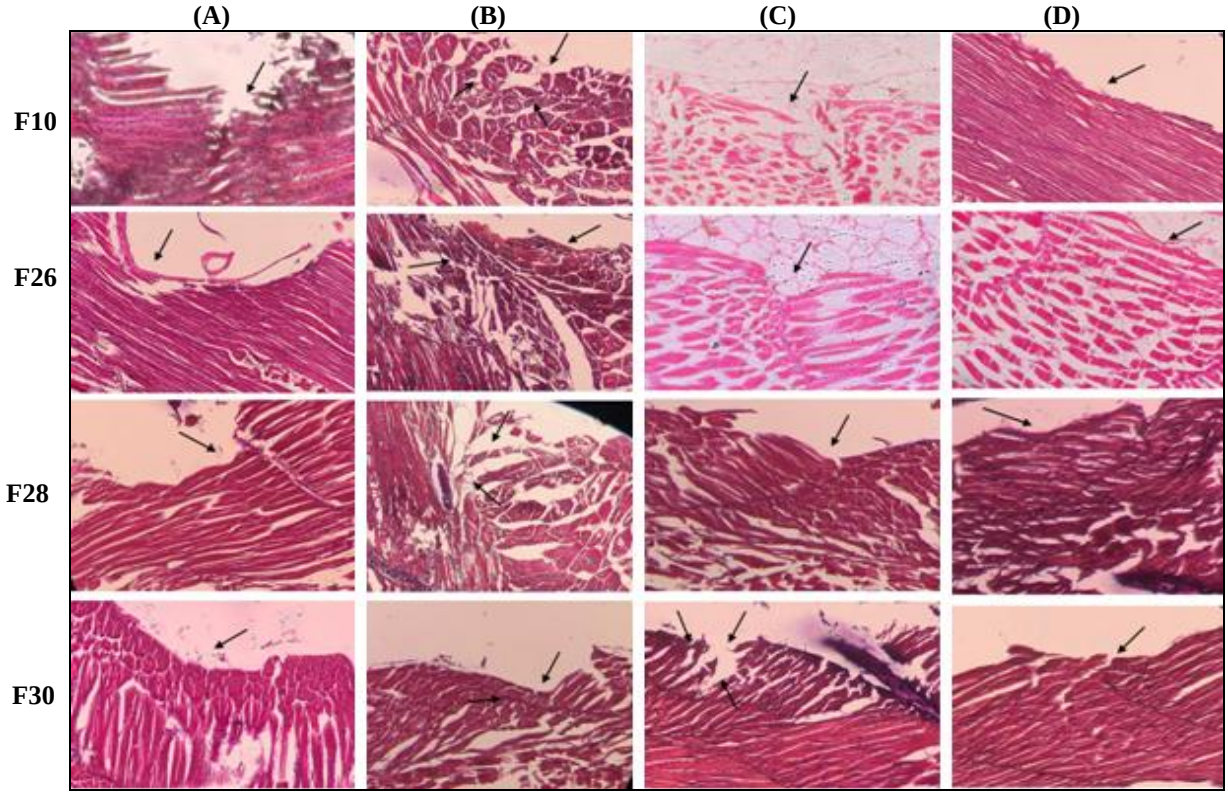


FIG. 4: NEUTROPHIL POPULATION AT THE WOUND SITE 0, 1 AND 3 DPW. AN INCREASE IN INFLAMMATORY CELLS AT 24 HRS AND EARLY RESOLUTION WAS OBSERVED IN F10 TREATED FISH IN COMPARISON TO THE CONTROLS. Values Are Expressed As Mean±Sd. The Results Obtained In Group Treated With F10 Were Highly Significant (P<0.05) When Compared With Control Group

Reepithelialisation and Granulation Tissue Formation: F10 treatment enhanced reepithelialisation and keratinocyte migration compared to the other groups **Fig. 5**. The F10 treatment promoted the reestablishment of the epithelial layer around 10 dpw, which was not seen in the untreated group. This effect correlates with the early resolution of inflammation by enhancing either neutrophil reverse migration or by inducing apoptosis by tissue macrophages. This might upregulate expression of growth factors and tissue proteins, to kick-start the proliferative phase that induces migration of the fibroblasts and keratinocytes through specific signalling mechanisms [38, 39]. Keratinocytes migration is mediated by release of cell adhesion proteins which later reform in the new site where the migrated cells establish a contact.

Glycitin, a soy isoflavone and 4, 6, 7-trimethoxyisoflavone at 200 µM concentration (1:1 ratio) synergistically induced dermal fibroblast proliferation and keratinocyte migration in mice excision wound model [40]. The combination is said to enhance secretion of TGF-β. Topical application of 10% ointments made from Hydromethanolic crude extracts of *Vernonia auriculifera* leaves on excision wound in mice reduced epithelialization period significantly from 21.17% to 17.83% [41]. The healing effects of *Calendula officinalis* were attributed to its stimulatory effect on fibroblasts migration and proliferation [42]. Fibroblasts either migrate from the nearby dermis or originate from fibrocytes, making up the major cells of the granulation tissue. Fibroblasts differentiate into myofibroblasts and contract the wound to remodel the collagen deposited to contribute at least 80% of the original tensile strength [43].



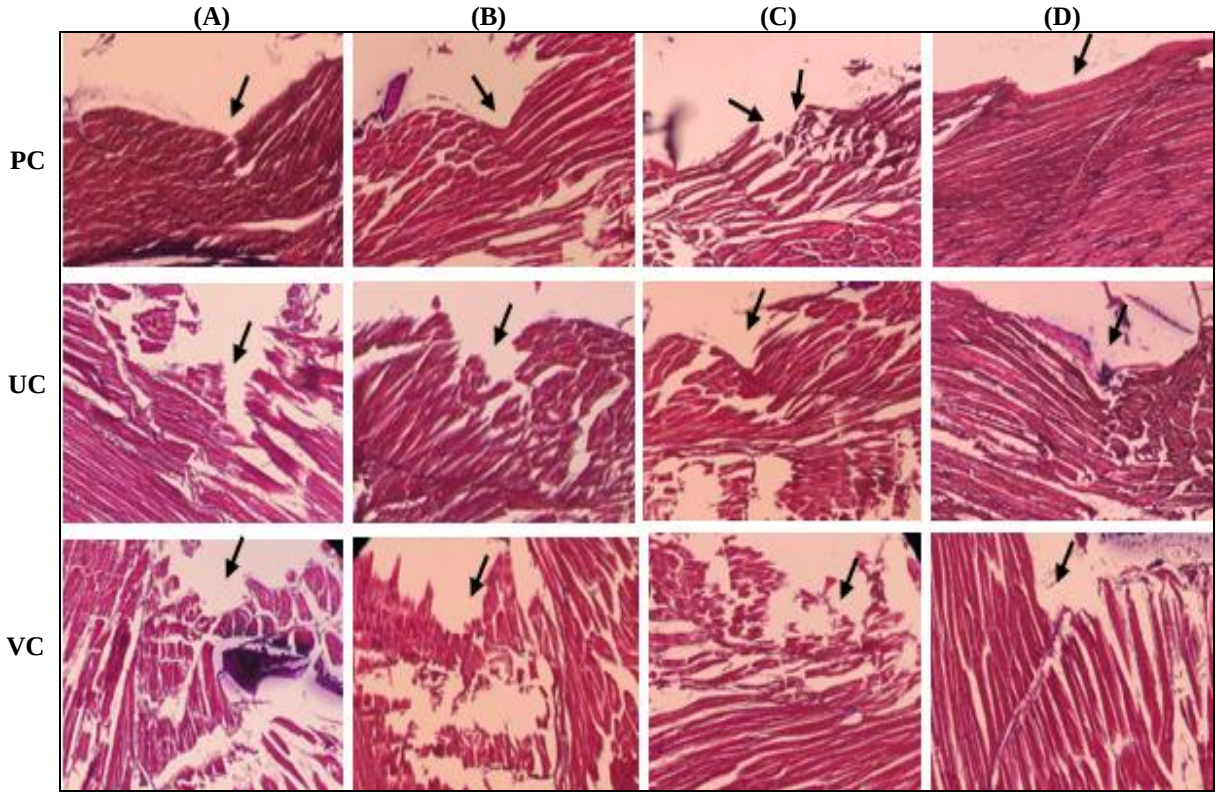


FIG. 5: H&E STAINED TISSUE SECTIONS OF FISH SHOWING THE MIGRATION OF CELLS AROUND THE WOUND TISSUE RESULTING IN WOUND CLOSURE; PANELS A, B, C AND D INDICATE OBSERVATIONS AT 0, 5, 7 AND 10 DPW. MIGRATION OF KERATINOCYTES AND EPITHELIAL CELLS FROM WOUND MARGIN WERE BETTER IN THE TREATED THAN THE CONTROL GROUPS AT 5 DPW. GRANULATION TISSUE FORMATION RESULTED IN A RE-STRATIFIED EPITHELIAL SURFACE AT THE WOUND SITE, 7 DPW. WOUNDED FISH WERE TREATED WITH TEST FRACTIONS (F10, F26, F28, AND F30); PC-POSITIVE CONTROL (TREATED WITH POVIDONE-IODINE OINTMENT 0.5%); UC-UNTREATED CONTROL, AND VC-VEHICLE CONTROL (TREATED WITH THE OINTMENT BASE). SCALE BAR = 30µM

Wet Weight and Total Protein Content of Granulation Tissue: The fraction treated groups showed high deposition of granulation tissue compared to the control, with a 40% increase in F10 treated tissues **Table 2**. The total protein content of the wet granulation tissue increased 35.67% more than the untreated control. Leaf extracts of *Vernonia arborea* have previously shown an increase in granulation tissue weight up to two times compared to the control group in Swiss Wistar rat wound models [6].

TABLE 2: GRANULATION TISSUE WEIGHT AND TOTAL PROTEIN CONTENT OF THE WOUND TISSUE OF THE ADULT ZEBRAFISH MECHANICAL WOUND MODEL [N=20] TREATED WITH TEST FRACTIONS F10-F30. TREATMENT WITH FRACTION F10 SHOWING HIGHER TOTAL PROTEIN CONTENT AND GRANULATION TISSUE WEIGHT THAN THE POSITIVE CONTROL

Group	Granulation tissue wet weight (mg/ g bw)	Total protein content (µg/ mg wet tissue)
Positive control	12.50±1.50	33.50±2.00
Untreated control	07.00±2.00	12.50±1.25
Vehicle group	08.07±1.25	14.00±1.65
F10	17.50±1.75	45.45±1.50
F26	08.80±0.65	15.50±1.35
F28	15.35±1.50	23.55±2.85
F30	14.54±1.80	40.50±1.75

Oxidative Markers: The concentration of reduced glutathione was found to increase from 5 to 7 dpw in treated fish as compared to untreated ones. The fish treated with fraction 10 showed increased GSSH concentration from 18.65 to 24.85 µM/mg tissue protein compared to untreated control **Fig. 6**, indicating cytoprotective role and balancing cellular redox potential. *Vernonia cinerea* extract at

500 µg concentration increased reduced glutathione from 4.37 to 5.94 nmole/mg protein in carrageenan induced mice paw oedema model [44]. On day 10 pw, the malondialdehyde concentration was found to reduce in treated fish compared to untreated control, the reduction being 6.8 times less after F10 treatment **Fig. 7**. This indicates the prevention of tissue damage by reducing tissue oxidative stress that leads to structurally and functionally intact tissue.

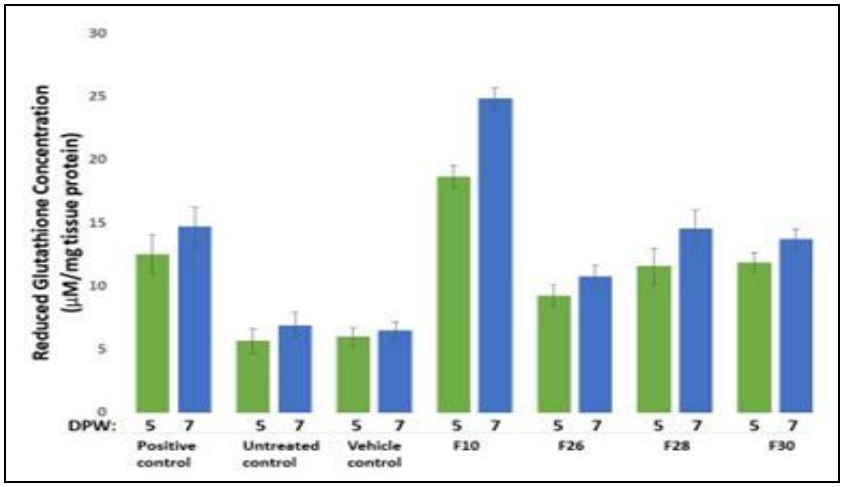


FIG. 6: REDUCED GLUTATHIONE CONCENTRATION IN THE TISSUE OF FORMULATION TREATED, CONTROL-TREATED AND UNTREATED ADULT ZEBRAFISH MECHANICAL WOUND MODELS [N=20] AT 5 AND 7 DPW. THE VALUES EXPRESSED ARE MEAN ± SD; F10 (0.5%) TREATMENT INCREASED REDUCED GLUTATHIONE 1.6 TIMES WHEN COMPARED WITH POSITIVE CONTROL

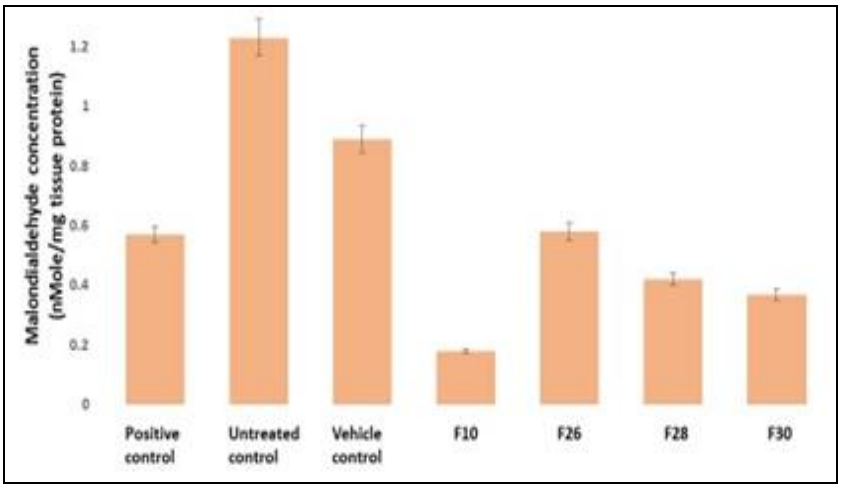


FIG. 7: MALONDIALDEHYDE CONCENTRATIONS IN WOUND TISSUES WERE REDUCED IN TREATED ADULT ZEBRAFISH [N=20 AT 10 DPW] AS COMPARED TO UNTREATED CONTROLS. AS COMPARED TO POSITIVE CONTROL, F10 TREATED WOUNDS SHOWED 3X DECREASE IN MALONDIALDEHYDE CONCENTRATIONS

Connective Tissue Markers: The degree of collagen formation in the healing tissue is indicative of regaining the structural integrity. The rate of collagen formation was studied in terms of hydroxyproline content in the tissue on days 7 and 10 pw. The fish treated with F10 showed increased hydroxyproline content on day 10 pw slightly greater than the positive control. The increase in hexosamine, one of the connective tissue markers, was determined across the control and treated groups on days 7 and 10 pw. A two-fold increase in hexosamine content in the healing tissue was prominent in the group treated with F10 as compared with the untreated control. The presence of hexuronic acid is significant for forming extracellular matrix components. Hence, an increase in hexuronic acid concentration indicates better connective tissue formation and efficient functional tissue restoration. The F10 treated group showed a better formation of hexuronic acid, with

52.16% increase as compared to positive control **Table 3**. Wistar Albino Rat wound models treated with 2% and 5% ethanolic extract of *Cestrum nocturnum* showed increased hydroxyproline concentration in granulation tissue after 10 dpw, indicating the elevated collagen content[12]. Topical application of the crude leaf extract of *Vernonia arborea* on Swiss Wistar rat wounds increased hydroxyproline content up to two times compared to the control [6].

TABLE 3: PROFILE OF TISSUE MARKERS AT THE WOUND SITE, OBSERVED AT DAY 7 AND DAY 10 POST-WOUNDING IN THE ADULT ZEBRAFISH MECHANICAL WOUND MODEL [N=20]. F10 WAS FOUND TO INCREASE THE MEASURED TISSUE MARKERS IN THE WOUND SITE DURING THE REEPITHELIALISATION PHASE, THEREBY INDUCING BETTER COLLAGEN DEPOSITION AND STABILIZATION FOR FASTER WOUND CLOSURE

Group	Hydroxyproline (µg/mg dry tissue)		Hexosamine (mg/100 mg dry tissue)		Hexuronic acid (µg/mg wet tissue)	
	Day 7pw	Day 10pw	Day 7pw	Day 10pw	Day 7pw	Day 10pw
Positive control	13.24±0.55	23.13±0.63	0.5±0.03	0.76±0.04	10.25±0.14	12.64±0.24
Untreated control	6.33±0.19	10.21±0.19	0.25±0.02	0.43±0.02	6.40±0.23	7.72±0.14
Vehicle group	7.27±0.24	12.41±0.07	0.31±0.04	0.55±0.01	8.56±0.12	10.40±0.14
F10	17.38±0.21	25.08±0.16	0.56±0.03	0.92±0.02	19.58±0.09	24.44±0.03
F26	15.44±0.12	19.43±0.15	0.44±0.02	0.66±0.02	13.50±0.08	15.17±0.15
F28	13.38±0.12	18.31±0.18	0.43±0.03	0.75±0.04	11.29±0.28	14.79±0.20
F30	15.01±0.02	23.43±0.15	0.47±0.02	0.86±0.02	15.43±0.22	17.73±0.12

A favourable profile of oxidative and tissue markers was observed in the wound model treated with F10. The values were highly significant statistically at $p<0.01$. This correlated with the early resolution of inflammation in this group when compared with the positive control.

Several phytocompounds have been tested for their wound healing potency and are understood to exhibit the property in various ways [45]. Acemannan, a mucopolysaccharide from *Aloe vera*, stimulates macrophages and induces transcription of proinflammatory mRNAs in healing wounds. Roots of *Astragalus propinguus* and *Rehmannia glutinosa* are reported to help diabetic wound healing by improving angiogenesis and reducing tissue oxidative stress in rat models. Wound dressing impregnated with extracts of *Azhadirachta indica* was found to exhibit anti-inflammatory and nitric oxide scavenging activity. *Panax ginseng* root extracts were studied to support keratinocyte migration and collagen deposition in human dermal fibroblasts [46].

In the albino rat excision wound model, *Vernonia amygdalina* leaf juice was shown to reduce leukocyte infiltration during early healing and enhanced fibroblast recruitment [47]. Two compounds, Vernolide and Isorhamnetin from the flower extract of this plant, showed antioxidant activity and were potent against *Staphylococcus aureus* [48]. The leaf fraction studied here showed better wound contraction, keratinocyte migration and proliferation, and enhanced expression of tissue markers that contribute to favourable collagen deposition and restore the lost tissue structure and function. This acceleration in the healing process could be attributed to the sequential pro- and anti-inflammatory properties of the phytocompound. The treatment was found to escalate neutrophil infiltration up to 1-2 dpw and succour resolution of inflammation 3 dpw. Thus, the favourable modulation of the inflammatory phase, augments the expression of pro- and anti-inflammatory cytokines during the early wound healing process and expedites the reepithelialization and tissue remodelling.

CONCLUSION: The cutaneous wound in Zebrafish created using the mechanical device delineates the multilateral tissue healing, that portrays the mammalian healing process. The model was used to assay for bioactive phytoconstituents that help the acceleration of wound healing. For the first time, Vernonia arborea fractions with antimicrobial and wound healing potencies are reported here. The wound healing potency of fraction 10 (0.5%) in this study was comparable to the standard povidone-iodine treatment and is supported by its ability to effectively reduce polymicrobial load at wound sites at 31.5 µg/Ml [7]. It promoted wound closure within short epithelialization times probably

because of its ability to balance the redox potential at the wound site that hampers tissue damage due to oxidative degradation. The rise in connective tissue components induced by the fraction 10 is higher than the povidone-iodine treatment, which encourages restoration of tissue integrity lost due to injury. Preclinical trials with other animal models would further validate the bioactivity of the formulated ointment for topical application on cutaneous wounds. However, the Zebrafish model described here is useful to screen a large number of extracts, compounds, and novel formulations for wound healing ability in compliance with the 3Rs of animal experimentation.

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