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Exploring the Wound Healing Potential of Piperine in Zebrafish *(Danio rerio)*: Insights from Visual Observations and Gene Expression Analysis

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KEYWORDS

Piperine; *Danio rerio*; Cutaneous wound; BAX; VEGF-1; MMP-2; HIF-1

ABSTRACT:

Introduction: A wound is a physical injury resulting in a break in the skin or underlying tissue, often caused by accidents, surgery, or disease. While medicinal plants have been recognized since time immemorial for their bioactive potential, evaluating their safety-to-efficacy ratio is critical. This study investigates the wound-healing potential and preclinical safety of piperine, the primary bioactive alkaloid in *Piper nigrum*, using a zebrafish (*Danio rerio*) model.

Objectives: The objective of this study was to evaluate the wound-healing efficacy of piperine in adult zebrafish through visual assessment of wound closure rates and to analyze the expression of angiogenic genes (*Bax*, *HIF-1*, *VEGF-1*, and *MMP-2*) to elucidate molecular mechanisms.

Methods: Adult zebrafish (n=24) were anesthetized with 0.2% Tricaine, and a single wound was created posterior to the abdominal region. The fish were divided into four groups (n=6): Group I (negative control/untreated), Group II (standard control/0.001 M doxycycline), and Groups III and IV treated with 0.001 M and 0.0001 M piperine, respectively. Piperine concentrations (0.001 M and 0.0001 M) were selected based on a determined LD_{50} of 60 µg/ml. Wound closure was monitored at 1, 4, and 7 days post-wounding (dpw) using a stereomicroscope and ImageJ software to calculate the wound healing percentage (WHP). On day 7, mRNA was isolated from muscle tissue for qRT-PCR analysis to quantify the relative expression of Bax, HIF-1, VEGF-1, and MMP-2. Statistical significance was determined using Student's t-test.

Results: Piperine treatment demonstrated dose-dependent healing, with the 0.001 M group achieving near-complete closure ($3 \pm 1.8\%$ residual area) by 7 dpw, representing a significant improvement over the negative control ($68 \pm 3\%$). Gene expression analysis revealed a marked upregulation of Bax, VEGF-1, and MMP-2, alongside a significant downregulation of HIF-1.

Conclusions: These findings highlight piperine as a promising and low-risk natural therapeutic agent for wound care acting through the modulation of angiogenesis-related gene expression.

1. Introduction

Skin is the largest human organ which is essential to maintain the body's homeostasis and balance. It serves as a physical barrier to prevent the loss of water and electrolytes and regulates the body temperature through sweating and blood flow changes (1). Physical injuries to the skin results in a disruption or break in the skin known as a cutaneous wound. In order to regain the skin's morphological continuity and functional condition, wounds must properly heal. Wounds are of two kinds, acute and chronic. Among these, a significant number of patients suffer from chronic wounds and the primary causes for chronic wounds are venous ulcers, pressure sores and trauma vasculitis. Furthermore, diseases like leprosy, diabetes mellitus, atherosclerosis, and tuberculosis can also lead to their development (2).

Wound healing is the complicated process involving various compounds such as soluble

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mediators, extracellular matrix, blood cells, and parenchymal cells. The process is divided into four main stages namely haemostasis, inflammation process, cellular proliferation, and tissue remodelling (3).

From time immemorial several plants have been identified for their wound healing potency. The bioactive compounds extracted from medicinal plants can promote growth factor secretion, shield tissue from oxidative stress, and eventually speed up the healing process (4). Black pepper (*Piper nigrum*), a member of the *Piperaceae* family, has received a lot of attention recently because of its rich phytochemistry and bioactivity (5). It primarily contains piperine, an alkaloid with several health advantages such as anti-inflammatory, anti-spasmodic, hepato-protective, anti-depressant, anti-bacterial, immunomodulatory, antifungal, antiapoptotic, anti-thyroid, anti-mutagenic, anti-metastatic, and anti-spermatogenic properties (6,7,8). Although piperine has several benefits, its potential as a wound-healing agent has not yet been thoroughly investigated. Therefore, examining piperine's ability to promote wound healing is the primary goal of the current study.

An efficient model system is necessary for evaluating the wound healing efficacy of natural compounds. The zebrafish (*Danio rerio*) has emerged as a valuable alternative due to its cost-effectiveness, rapid tissue regeneration, and conserved biological principles (9). This study utilizes the zebrafish model to investigate the wound-healing efficacy of piperine.

In the present study we have visually analysed the wound closure of piperine treated zebra fish after 1, 4 and 7th day post wounding. Angiogenesis is vital for the proliferative phase of healing and is regulated by a balance of inhibitors and growth factors, such as Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), which stimulate endothelial cell migration and tissue repair. Thus, the study also explored the expression of angiogenesis-related genes BCL2-associated X protein (Bax), Hypoxia Inducing Factor-1 (HIF-1), Vascular Endothelial Growth Factor-1(VEGF 1) and Matrix Metalloproteinase-2 (MMP-2) in treated zebrafish on the 7th day post-wounding.

2. Methods

2.1 Procurement of pure compound Piperine and preparation of stock solution

Pure compound Piperine was purchased commercially (Sigma, Aldrich). To prepare a stock solution, exactly 2.853 g of piperine was dissolved in 50 mL ethanol and diluted to 1 litre using distilled water, resulting in a concentration of 0.01 M. Two different concentrations, 0.001 M and 0.0001 M, were prepared by diluting the stock solution. These concentrations were chosen in the present study based on the 50 % lethal dose of toxicity (LD_{50}) concentration of piperine to zebra fish. LD_{50} of piperine was at a concentration of 60 μg/ml.

2.2 Culture and Maintenance of Zebrafish

The wild type zebrafish were obtained from a commercial aquarium located in Chennai, Tamil Nadu, and they were kept under standard laboratory conditions at a temperature of 28 °C in a 12-hour light and 12-hour dark cycle. The fish were housed in an automated water circulation system with a stocking capacity of 10 fish per 3.5 litre aquarium tank. The fish were fed with brine shrimp three times daily, at 4 % of their body weight. Healthy fish that were consistent in size and around 6 - 7 months old were selected for the experiment.

2.3 Wounding and Piperine treatment

All the fish were anaesthetized by immersing in 0.2% Tricaine (ethyl-3-aminobenzoate methane-

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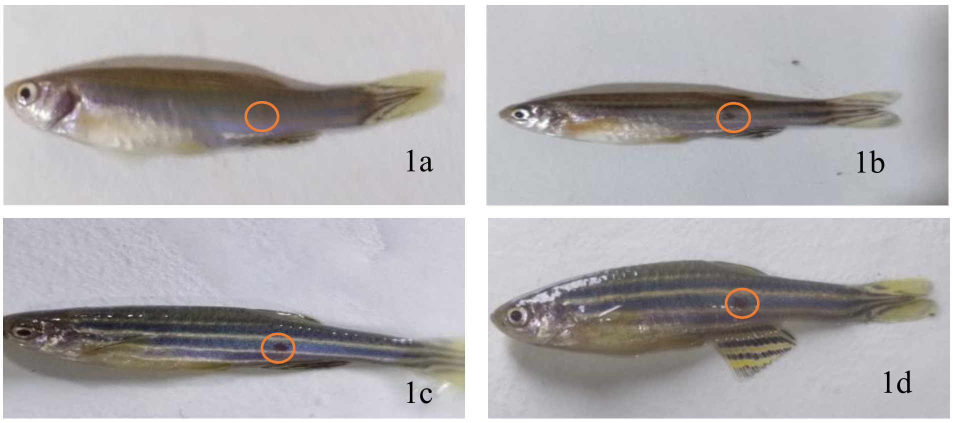
sulfonate) (Sigma-Aldrich). Following anaesthesia, a single wound was created by a sharp scalpel, posterior to the abdomen regional area, close to the lateral line of the zebrafish.

To determine the wound healing percentage, the zebra fish were divided into four groups of 6 (n= 24). Group I (Fig 1a) was negative control (wounded- untreated), Group II (Fig 1b) standard control (treated with 0.001 M doxycycline), Group III (Fig 1c) (treated with 0.001 M Piperine), and Group IV (Fig 1d) (treated with 0.0001 M Piperine). On zero-day post wounding (dpw) the photographs were taken for all the four groups (Figs. 1a- 1d). The wounded fish of all the four groups were visually observed on 1 dpw, 4 dpw and 7 dpw.

measured at different time points. The wounded area was identified based on the difference in skin colour between wounded and unwounded tissue; when the area was no longer distinguishable, the wound was considered healed. The wound healing effect of piperine (WHP) was calculated by comparing the difference in wound size between 1 dpw and the subsequent days (4 and 7 dpw), expressing this as a percentage relative to the 1 dpw wound size.



Figures 1a – 1d Photographs of Zebrafish on 0th day post wounding

2.4 Visual Observation and Determination of Wound Healing Percentage

On 1, 4 and 7dpw, when zebrafish were under anaesthesia, the wounds of 6 fish per group were photographed, using a digital camera attached to a stereo microscope (Nikon-SMZ-100, Japan). Then the wound area was measured by Image J software (ver.148, USA). After taking a photo of the caudal fin, each fish was uniquely recognised by comparing its pigmentation pattern to the pattern on the fin. The wound area of individual fish was

2.5 Quantitative real time polymerase chain reaction (qRT-PCR) analysis for genes involved in angiogenesis

For the gene expression analysis, the same experimental set up was established viz., Group I was negative control (wounded-untreated), Group II standard control (treated with doxycycline 0.001 M), Group III (Piperine, 0.001M), and Group IV (Piperine,0.0001M).

2.5.1 Isolation of RNA and synthesis of cDNA

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Muscle tissues were collected from three zebrafish per group at 7 days post-wounding (dpw). Tissue samples were immediately snap frozen in liquid nitrogen and kept at -80ºC. For RNA extraction, 200 mg of tissue pooled from the three fish in each group was processed using TRIzol reagent (Invitrogen, USA).

Pooled RNA (2.5µg) was used for cDNA synthesis using a PrimeScript 1st strand cDNA synthesis Kit (TaKaRaR, Japan), as per the protocol given by the manufacturer. Using nuclease-free water synthesized cDNA samples were diluted 30 times and stored at -20 ºC for further study.

2.5.2. Gene Expression Analysis

In the present study, representatives of angiogenesis related gene viz. Bax, Hypoxia Inducing Factor-1(HIF 1), Vascular Endothelial Growth Factor-1(VEGF 1) and Matrix Metalloproteinase-2 (MMP-2) were analyzed using qRT-PCR in a TaKaRa Thermal Cycler Dice TP 800 real-time system. The gene-specific primers are listed in Table 1.

The total volume of reaction mix was 10 µl which contained 4 µl of cDNA, 5 µl of 2 X TaKaRa Ex-Taq™ SYBR premix and 0.5µl of each forward and reverse primer (10µM). The standard three-step thermal cycling profile of the machine with 55ºC annealing, followed by a single dissociation reading step at the end, was performed to identify the specificity of the primers. The relative expression fold was determined by the method described by Livak and Schmittgen (2001) (11).

2.6 Statistical Analysis

All the experiments were done in triplicates (n=3) and the sample mean was calculated. The data are represented as mean ± standard deviation. The results of the various measurements between the treated and untreated groups were statistically quantified using Student's t-test.

Table 1. Prime information of zebra fish genes selected for present study

Gene Name	Accession Number	Primer Name	Primer Sequence (5'→3')
bax	NM_131562.2	Bax- F Bax-R	GTGGTACGACCAGAGGCATAC AAGGCCAACAGGGAAAAGAT
hif-1	NM_001308559.1	HIF-1 F HIF-1 R	AGCCGCCACACTTTAGACAT CCTCTGGATCAAAACCCAAG
vegf-1	NM_001110349.2	VEGF-F VEGF-R	TGCTCCTGCAAATTCACACAA ATCTTGGCTTTTCACATCTGCAA
mmp-2	NM_198067.1	MMP-2-F MMP-2-R	CCTCACGATCCTTCTGGCTT AATTCTGCTTCACAATGATA

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The present study deduced the wound healing efficacy of Piperine in zebrafish (*Danio rerio*). Based on the previous experiments carried in our laboratory, the LD_{50} toxicity of piperine was determined as 60 µg/ml.

3.1 Effect of Piperine on Wound healing

To evaluate the wound-healing properties of Piperine, the size of the wound was visually assessed on days 1, 4, and 7 post wounding (dpw). Due to poorly defined wound margins immediately after the injury (0 dpw), it was not possible to measure the wound area accurately at this time point.

Fig. 2 Group I (0th, 4th and 7th day post wounding**)**

Fig. 3 Group II (0th, 4th and 7th day post wounding)

Fig. 4 Group III (0th , 4th and 7th day post wounding)

Fig. 5 Group IV (0th, 4th and 7th day post wounding

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Therefore, the initial evaluation of the wound size was performed on day 1 post-wounding, and the wound area was measured at that time point, followed by additional assessments on days 4 and 7 post-wounding (dpw).

Immersion treatment of piperine displayed a dose-dependent wound closure when compared to the negative control (wounded -untreated). The visual images of all the four groups are presented in Figs. 2-5.

The wound-healing efficacy of piperine was assessed by calculating the percentage of the wound area on days 1, 4, and 7 post-wounding (dpw), relative to the initial wound size (Day 0), as shown in Table 2 and Fig. 6. The results demonstrate a gradual decrease in the wounded area across all experimental groups over the 7-day observation period.

Table 2 Percentage of Wounded Area after 1, 4 and 7th Day Post Wounding

Experimental Groups	1 dpw (Mean % ± SD)	4 dpw (Mean % ± SD)	7 dpw (Mean % ± SD)
Group I	100 ± 2	83 ± 4	68 ± 3
Group II	100 ± 3	39 ± 2	2 ± 0.8
Group III	100 ± 3	43 ± 4.2	3 ± 1.8
Group IV	100 ± 3	78 ± 3.2	37 ± 2.1

*Wound healing effect of piperine among the different concentrations are significantly different for each day post wounding (p<0.05)

**Wound area among the different experimental is significantly different for each day post wounding (p<0.05).

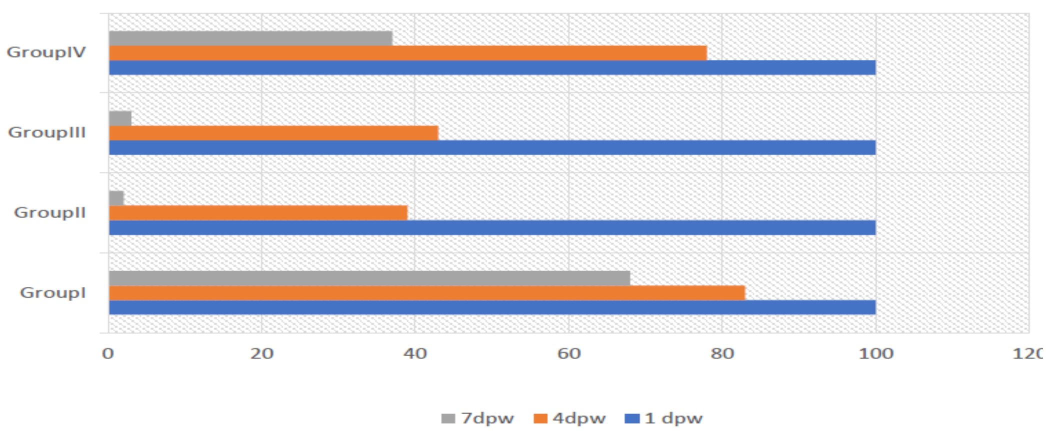

Fig. 6 Percentage of wound Area on 1, 4 and 7 day post wounding (dpw)

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On day 4, the percentage of the remaining wounded area was 83%, 39%, 43%, and 78% for groups I, II, III, and IV, respectively. By day 7, a significant acceleration in wound closure was observed in the treated groups compared to the negative control. Specifically, the residual wound area for groups I, II, III, and IV was 68%, 2%, 3%, and 37%, respectively. Notably, groups II (Doxycycline) and III (0.001 M Piperine) showed the marked reduction in the wounded area, nearly achieving complete wound closure by the end of the study period.

Table 3 Relative mRNA expression of angiogenetic genes

GENES	Group I	Group II	Group III	Group IV
BAX	13.71±0.95	24.81±1.95	25.71±2.6	26.73±2.0
HIF-1	30.78±1.4	23.54±1.14	22.35±2.05	23.64±2.08
VEGF1	19.01±1.97	26.04±2.62	25.95±1.29	25.43±1.29
MMP-2	21.34±1.53	23.68±1.99	24.61±3.36	24.54±3.36

3.2 Transcriptional analysis of selected genes during wound healing upon treatment with Piperine

Relative mRNA expression of the genes viz. Bax, Hypoxia Inducing Factor-1(HIF 1), Vascular Endothelial Growth Factor-1(VEGF 1) and Matrix Metalloproteinase-2 (MMP-2) of adult zebra fish after 7 dpw in all the four groups are presented in table 3 and Figs. 7a – 7d.

3.2.1 Bax gene

The proteins Bcl-2-associated X protein (Bax), which promotes apoptosis, and B-cell lymphoma 2 (Bcl-2), which inhibits it, are pro- and anti-apoptotic, respectively. These proteins maintain the structure of healthy proteins while repairing or removing defective proteins. They are necessary to maintain cellular homeostasis in spite of changes in physiological and environmental factors. The results of this study indicated that positive control, doxycycline in administration resulted in the increased Bax expression compared to negative control group. Similarly, piperine also enhanced the Bax expression in dose dependent manner. Bax mRNA expression was more in groups II, III and IV, when compared with group I (wounded-untreated) (Fig . 7a) . The gene was upregulated by 2 and 2.5 fold in groups treated with 0.001 and 0.0001 M concentration of piperine. However, expression of Bax was lesser in wounded-untreated group (negative control).

3.2.2 Hypoxia Inducing Factor-1(HIF- 1)

HIF-1 significantly influences the rate and efficiency of tissue repair as it is the primary mediator of the cellular response to hypoxia. Hundreds of downstream target genes, many of which play crucial roles in the process of wound healing, are induced to express by the transcription factor HIF-1 when hypoxia occurs. HIF-1

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expression was high in group I (wounded-untreated) when compared to all the other groups (Fig.7b). The expression of this gene was reduced to half fold in doxycycline treated group compared to the wounded controls. In 0.001 and 0.0001 M piperine treated groups the exhibited reduced expression were approximately 0.6 and 0.75 fold respectively.

3.2.3 Vascular Endothelial Growth Factor-1 (VEGF1)

VEGF-1 performs the roles of a chemotactic agent, an inducer of vascular permeability, and an endothelial cell mitogen. VEGF is distinct due to its effects on numerous stages of the wound healing cascade, including angiogenesis. The mRNA expression of VEGF-1 was higher in groups II, III, and IV compared to group I (wounded-untreated) (as shown in Fig.7c).

The expression of the gene, VEGF-1 in 0.001 and 0.0001 M of piperine showed 1 and1.5fold higher expression than the control group. However, in the wounded-untreated group (negative control), the expression of VEGF-1 was significantly less.

3.2.4 Matrix Metalloproteinase-2 (MMP-2)

The main tissue remodelling enzyme matrix metalloproteinases-2 (MMP-2) play several overlapping roles in wound healing and tumour growth. The expression of MMP-2 in the muscle of zebrafish in all four groups after 7 days post-wounding (dpw) is depicted in Fig.7d. The expression of this gene was higher in groups II, III, and IV, whereas it was significantly lower in the wounded-untreated group.

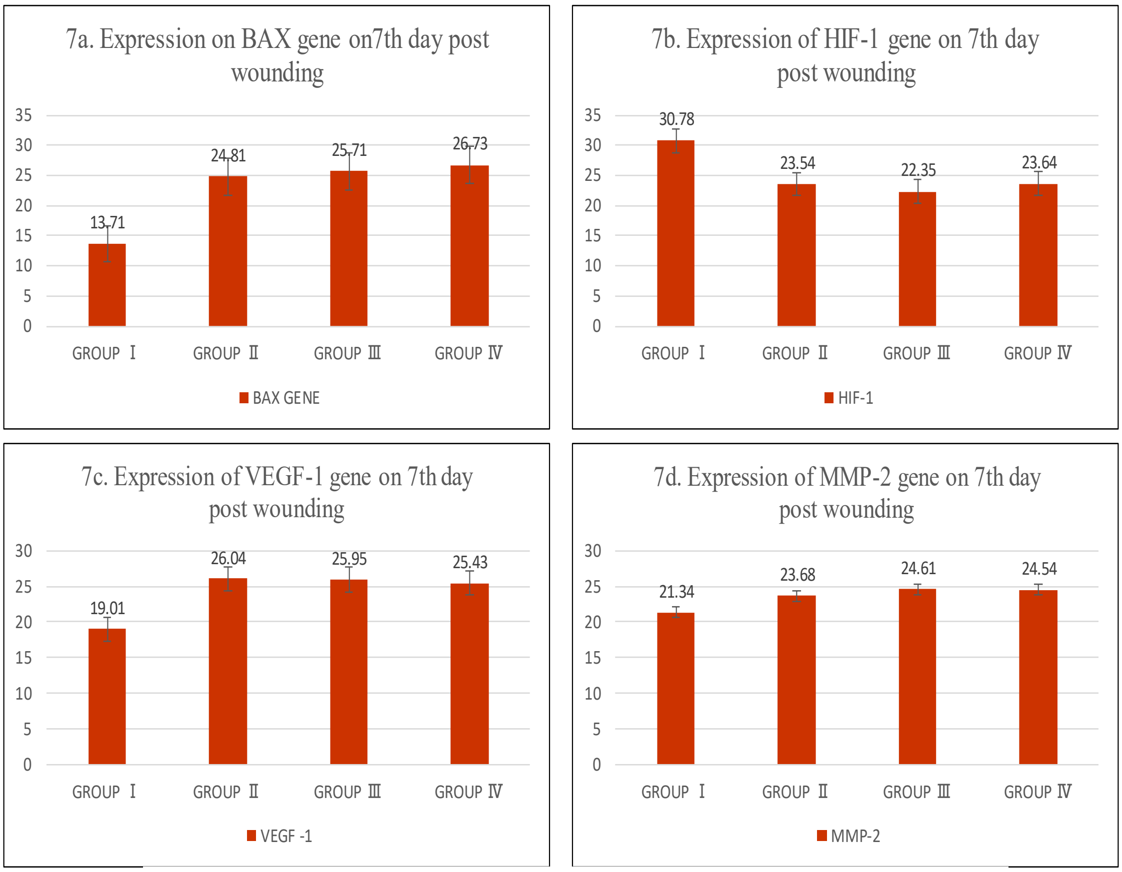

Fig. 7a – 7d Expression of genes on 7th day post wounding

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4.Discussion

Zebrafish (*Danio rerio*) has become a significant experimental model for investigating wound healing owing to its exceptional regeneration abilities, transparent embryos, and genetic accessibility. These capabilities provide real-time visualisation of wound healing processes, including immune cell migration, angiogenesis, and cellular proliferation, yielding significant mechanistic insights (12, 13). Zebrafish skin's structural resemblance to human skin confirms that it is suitable for research on cutaneous wound healing (14).

This study examined the wound healing capability of the bioactive alkaloid piperine, extracted from black pepper (*Piper nigrum*), in zebrafish. Piperine possesses established anti-inflammatory and antioxidant characteristics and has demonstrated effectiveness in facilitating collagen repair and regulating essential signalling pathways in rodent models (15). However, more research was needed to determine its function in cutaneous wound healing.

According to our findings, zebrafish wound repair was considerably improved by immersion treatment with piperine at doses of 0.001 M and 0.0001 M when compared to untreated controls. Visual evaluation and quantitative measurements of the wound area at 4 and 7 days after wounding were used to verify this effect. Prior reports of piperine-enhanced tissue regeneration in mammalian models are consistent with the observed acceleration of wound healing (16).

Bax, VEGF-1, and MMP-2 are essential for apoptosis, angiogenesis, and extracellular matrix remodelling, respectively, and their expression was elevated at the molecular level by piperine treatment. A higher expression of Bax indicates eradication of injured cells, which speeds up the process that produces effective tissue healing. Increased VEGF-1 levels signify angiogenesis, which is vital for providing regenerated tissues with nutrition and oxygen. MMP-2 upregulation promotes active matrix remodelling, which facilitates wound contraction and fibroblast migration (17,18). In contrast to wounds that remain untreated, piperine-treated groups showed a decrease in HIF-1 expression, suggesting better tissue oxygenation and less hypoxic stress. The enhanced healing results observed are probably a result of this control over hypoxia-related pathways.

5. CONCLUSION:

In conclusion, the current study indicates that piperine has the potential to improve cutaneous wound healing through the modulation of important biological pathways related to matrix remodelling, angiogenesis, and cell death. These results establish the basis for more preliminary research and the development of piperine-based wound care treatments.

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